Volume IX Number 2

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THE UNIVERSITY OF CHICAGO PRESS
CHICAGO, ILLINOIS, U.S.A.

PHYSIOLOGICAL ZOÖLOGY

A Quarterly Journal of Zoölogical Research

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Phyziological Zoölogy is published quarterly by the University of Chicago at the University Press, 5750 Ellis Avenue, Chicago, Ill. The subscription price is $7.50 a year; the price of single copies is $2.25. Orders for service of less than a half-year will be charged at the single-copy rate. Postage is prepaid by the publishers on all orders from the United States, Mexico, Cuba, Porto Rico, Panama Canal Zone, Republic of Panama, Dominican Republic, Canary Islands, El Salvador, Argentina, Bolivia, Brazil, Colombia, Chile, Costa Rica, Ecuador, Guatemala, Honduras, Nicaragua, Peru, Hayti, Uruguay, Paraguay, Hawaiian Islands, Philippine Islands, Guam, Samoan Islands, Balearic Islands, Spain, and Venezuela. Postage is charged extra as follows: for Canada and Newfoundland, 15 cents on annual subscriptions (total $7.65), on single copies 4 cents (total $2.29); for all other countries in the Postal Union, 40 cents on annual subscriptions (total $7.90), on single copies 10 cents (total $2.35). Patrons are requested to make all remittances payable to The University of Chicago Press in postal or express money orders or bank drafts.

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For China: The Commercial Press, Ltd., 211 Honan Road, Shanghai. Yearly subscriptions, $7.50; single copies, $2.25, or their equivalents in Chinese money. Postage extra on yearly subscriptions 40 cents, on single copies 10 cents.

Claims for missing numbers should be made within the month following the regular month of publication. The publishers expect to supply missing numbers free only when losses have been sustained in transit and when the reserve stock will permit.

Business correspondence should be addressed to The University of Chicago Press, Chicago, Ill.

Communications for the editors and manuscripts should be addressed to Charles M. Child or W. C. Allee, Managing Editors of PHYSIOLOGICAL ZOÖLOGY, The University of Chicago, Chicago, Ill.

The articles in this journal are indexed in the *International Index to Periodicals*, New York, N.Y.

Applications for permission to quote from this journal should be addressed to The University of Chicago Press, and will be freely granted.

Entered as second-class matter October 27, 1928, at the post-office at Chicago, Illinois, under the Act of March 3, 1879.

[PRINTED IN U.S.A.]

Physiological Zoölogy

Vol. IX APRIL 1936 *No. 2*

THE PHYSIOLOGY OF THE FORMATION OF "PIGEON'S MILK"[1]

(Two plates)

M. D. PATEL
Bombay Gowrakshan Cattle Farm, India

IT HAS long been known that males, as well as females, of pigeons and doves produce in their crop a milky substance, commonly known as "pigeon's milk," which is fed the young for the first few days after hatching. Investigations on the subject have been, in the main, of a histological and cytological nature. Only recently have attempts been made to discover the factors inducing the crop changes and the mechanism by which they are brought about. The investigations described in this paper deal with the changes in the crop under various experimental conditions.

The pigeons used in these investigations were from the stock maintained by the Department of Genetics of the University of Wisconsin for studies on inheritance.

For histological examination of the crop Bouin's fluid was found more satisfactory than Zenker's. Not only was Bouin's easier to handle, but more precise fixation and staining were possible by its

[1] "Papers from the Department of Genetics, Agricultural Experiment Station, University of Wisconsin," No. 175. Published with the approval of the director of the Station. Aided by a grant from the Committee for Research in Problems of Sex of the National Research Council, grant administered by Professor L. J. Cole. Submitted to the faculty of the University of Wisconsin in partial fulfilment of the requirements for the degree of Doctor of Philosophy. The writer gratefully acknowledges his indebtedness to Dr. L. J. Cole for valuable suggestions and criticisms throughout the work. Thanks are also due Dr. M. R. Irwin for valuable assistance given.

use. For general staining Harris' haematoxylin was most frequently used, though Delafield's also gave good results. Heidenhain's iron haematoxylin was employed for a more specific study of the nucleus. For the relation of connective tissue to other tissues Mallory's triple stain was used. Flemming's strong solution, followed by safranin, was employed to differentiate special cytoplasmic structures, especially fat droplets.

THE NORMAL HISTOLOGICAL CHANGES IN THE CROP DURING INCUBATION AND FEEDING OF THE YOUNG

The histological changes in the crop, especially during incubation, are presented exhaustively by Litwer (1926) and Beams and Meyer (1928, 1931). A brief review is necessary, however, in order to stress some of the important changes which have been used as landmarks in judging crop growth obtained under experimental conditions.

The wall of the crop in the non-brooding bird is thin, transparent, and of yellow-white color, and consists of four coats: the outer (basal) fibrous, the muscular, the submucosa, and the mucosa (Plate I, Fig. 1). The outer, loose fibrous tissue envelops the wall and unites it to the adjacent organs. The muscular coat contains an outer longitudinal and an inner circular layer of muscle fibers. The submucosa consists of areolar connective tissue with many blood vessels and is covered by a layer of loose connective tissue, tunica propria, containing irregularly branched blood vessels, from which the mucous coat draws its nourishment. The deep layers, stratum germinativum, of the stratified epithelium adjacent to the tunica propria, consist of young, columnar, deeply staining cells, the significance of which in the hypertrophy of the crop during incubation is justly pointed out by Litwer (1926). The line of demarcation between the stratum germinativum and the tunica propria is not uniform but shows recurring smooth and zigzag areas; the latter are due to the protrusions of tunica propria into the stratum germinativum. This condition is, however, absent in the sections of the median portion of the crop, and it is suggested by Litwer that this may account for the fact that this part of the crop remains unaltered during incubation. The epithelial cells of the crop at this stage, fixed in Flemming's solution, are invariably free of lipoid droplets. This structure of the crop persists during the first 7 days of incubation.

From the eighth to the thirteenth day the walls of the lateral pouches manifest marked macroscopic and microscopic changes. Particularly by the twelfth day there is an increase in vascularity and a slight hypertrophy of the wall. Folds are noticeable even on the eighth day of incubation (Plate I, Fig. 2), and by the twelfth day their size is remarkably increased. Sections show an augmentation in the connective tissue and vascularity of the protrusions of the tunica propria into the folds of the stratum germinativum.

With the further development of the folds, accompanied by thickening of the epithelium, the spaces between them become obliterated (Plate I, Fig. 4, and Plate II, 10), while secondary ramifications of the tunica propria into the stratum germinativum, carrying capillaries with them, result in a structure superficially glandlike in appearance (cf. Plate II, Fig. 7).

The hypertrophy at this stage is accordingly brought about largely by the excessive cell multiplication in the stratum germinativum. By the eighth day lipoid droplets begin to appear in the epithelial cells and increase as the hypertrophy continues. In the meantime the median portion of the crop remains unchanged.

The incubation period of the pigeon is about 18 days. From the thirteenth day of incubation to the fourth day after hatching of the young the changes in the crop proceed with remarkable rapidity, as is evident from Plate I, Figures 3–5. The lateral pouches are rosy red in color, owing to increased vascularity, and exhibit extraordinary hyperplasia, which can be felt by palpation through the skin. The "milk" is found in the crop on about the seventeenth day of incubation. During this period the histological changes are more marked than those in the preceding period, and consist, generally, in an enormous hypertrophy of the epithelium (Plate I, Figs. 3, 4), in an increase in size and number of lipoid droplets in the hypertrophied cells, and finally in a desquamation of the surface cells of the thickened epithelium. The rate of cell multiplication in the stratum germinativum further increases, and there is an indication that the cells of the intermediate layers may divide amitotically. This is presumably associated with an early stage of degeneration due to a scarcity of nutriment, since these cells are relatively at a greater distance from the source of nutrition. The distribution of lipoid droplets in the cells of the thickened epithelium is uneven; the young columnar

PLATE I

(Photomicrographs)

FIG. 1.—Section of the wall of a lateral pouch of the crop of a non-brooding bird. The basal layer is the fibrous (*fibr.*); next the muscular (*musc.*) showing bundles of both longitudinal and transverse fibres; the areolar submucosa (*sub. mu.*) with its covering of vascular tunica propria (*t. p.*), which supplies nourishment to the mucosa, consisting of the more deeply lying stratum germinativum (*str. ger.*) and the stratified epithelium (*ep.*), lining the lumen of the crop. Note that the epithelium is wrinkled but not definitely folded. ×50.

FIG. 2.—Section of the pouch wall on the eighth day of incubation. The epithelium is thrown into deep folds. Note the greater stratification of the epithelium and the vascularity of the protrusions of tunica propria into the epithelial folds. The increased thickness of the crop wall is at this stage due almost entirely to the depth of the folds. ×50.

FIG. 3.—Section of pouch wall at fourteenth day of incubation. The epithelium has increased greatly in thickness. ×50.

FIG. 4.—Section of the pouch wall on the third day of feeding of the young. Here there is still greater hypertrophy of the epithelium, and the process of desquamation of epithelial cells (liberation of "milk") is evident. ×20.

FIG. 5.—Enlarged view of portion of Figure 4 showing the sloughing-off of masses of epithelial cells. ×125.

FIG. 6.—Section of "milk" from the crop of a young squab. Note that the outlines of the nucleated epithelial cells are still evident. ×125.

PLATE II

(Photomicrographs)

FIG. 7.—Section of the crop of ♂2348A on the nineteenth day of incubation. This bird was prevented from sitting on the eggs from the fifth day of brooding onward but was in sight of his mate during that time. The crop hypertrophy is normal for full incubation (cf. Figs. 3 and 4). ×20.

FIG. 8.—Section of the crop of ♂1743R on the nineteenth day of incubation. The actual time of incubation was, however, cut down by three periods of removal. This retarded the crop development, which approximates that of the tenth or eleventh day of normal uninterrupted incubation. ×50.

FIG. 9.—Section of the crop of the same bird (♂1743R) on the twenty-fifth day of prolonged incubation. The development has reached the stage normally attained at the fourteenth day (cf. Fig. 3). ×50.

FIG. 10.—Section of crop of same bird (♂1743R) on twenty-ninth day of incubation. Epithelium greatly thickened and desquamation commencing. Compare with the normal situation after 3 days of feeding young (Fig. 4). ×20.

FIG. 11.—Section of testis of ♂2501G on sixteenth day of incubation. Shows normal stages of spermatogenesis, with no indication of quiescence or regression. ×250.

PLATE I

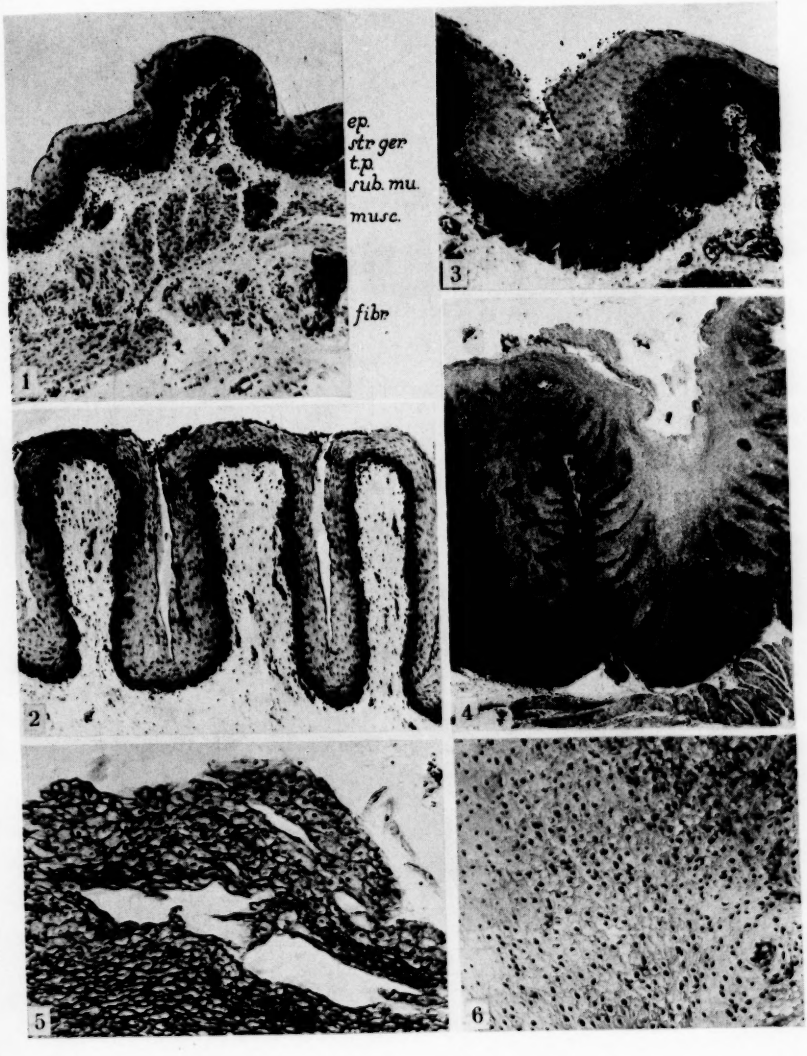
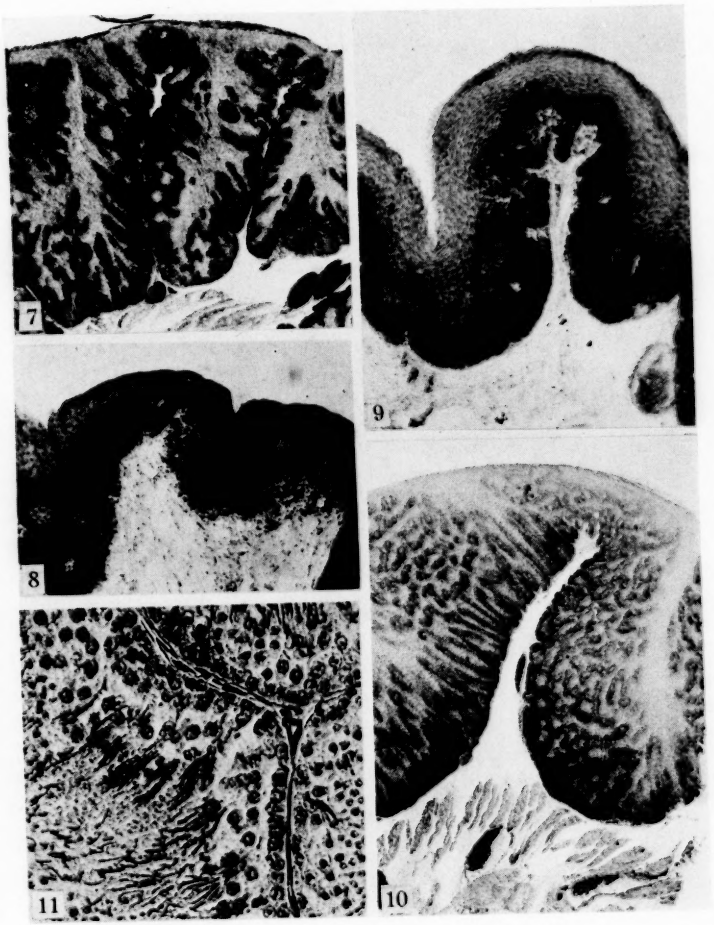


PLATE II



cells of the stratum germinativum are free of these droplets, while the cells nearest to the lumen of the pouch contain more and larger lipoid droplets than those farther away. As a result of increased growth of the epithelium of the folds, the space occupied by the connective tissue and the blood vessels of the tunica propria in the fold is obliterated to a mere passage for the blood vessels (Plate I, Fig. 4).

The desquamation of the surface epithelial cells occurs over the entire area of the pouches and usually involves groups of relatively few cells. It is not uncommon, however, for several surface layers of the epithelial cells to slough off into the lumen in large masses (Plate I, Figs. 4 and 5). The histological picture of the "milk" obtained from the crop of a squab (Plate I, Fig. 6) approximates that of the epithelial cells which are ready to slough off (Plate I, Fig. 5). In both cases the cells are polygonal, with flattened nuclei, around which are vacuoles representing dissolved lipoids.

The processes of growth and desquamation in the pouches of the crop continue until about the eighth or ninth day of feeding, following which the crop returns to normal by the fourth week of feeding. Marked hyperplasia of the epithelium still exists even on the sixteenth or seventeenth day of feeding, but no "milk" is found in the crop.

With reference to the glands of Teichmann (1889), located where the crop joins the lower part of the esophagus, our observations indicate that they are functionally active even outside the feeding period. While these glands may supply enzymes (Dulzetto, 1927*a*, 1927*b*, 1928*a*, 1928*b*) or amino acids (Carr and James, 1931), their increased secretion of mucus during the feeding period probably aids also in the regurgitation of the milk.

RÔLE OF INCUBATION IN MILK FORMATION

The facts presented in the preceding section indicate that the formation of the milk in pigeons is due to physiological factors associated with incubation. The *modus operandi* of the incubation instinct and the nature of the physiological processes underlying it are, however, little understood. An experimental procedure, therefore, to determine the rôle of incubation in the formation of the milk should consist in modifying the act of incubation and in observing the effects of such alterations on the phenomenon.

In pigeons the reproductive cycle consists of five discrete terms, which Herrick (1907) groups as cyclic instincts. These instincts follow in a definite sequence; and if the cycle is broken at any point, the whole process is abandoned and the bird begins the cycle anew. The incubation instinct is preceded by the instinct of courtship and mating, of nest-building, and of egg-laying; while the instinct of feeding and care of the young follows it.

A comprehensive description of the normal incubation behavior in pigeons is presented by Whitman (1919). The incubation period —from the time of the laying of the first egg to the hatching of the young—is 17–19 days. The male, however, does not commence incubating regularly until the appearance of the second egg. After the laying of the second egg both birds participate regularly in the act. At night the female sits on the eggs, while the male roosts away from the nest. During the day the male and female alternate in the act; the male ordinarily occupies the nest from about 10:00 A.M. to 4:00 P.M. The initiative in leaving or resuming the nest is taken by either bird. When the nest is relinquished by one bird, this acts as a signal for the other bird to resume the nest duty, and this is done immediately.

SEPARATION OF PARENTS DURING INCUBATION

EXPERIMENTS 1 AND 2: SEPARATION OF ONE BIRD OF PAIR TO ADJACENT CAGE BUT WITH VIEW OF MATE

In Experiment 1 the males of nesting pairs were separated from the females at different stages of incubation and placed in pens adjacent to their mates, with only a wire-netting partition between. In Experiment 2 the females were removed in a similar manner. The separated bird and the brooding mate were thus within sight of each other.

In Table I will be found a record of Experiment 1. It will be seen that, although the males were prevented from incubating eggs for 7–16 days, in all cases the normal crop changes were obtained. Plate II, Figure 7, shows the histological picture of the crop of ♂2348A at the end of the normal incubation period. The changes are similar to those obtained at the same time in birds that have incubated normally for the same period (cf. Plate I, Fig. 4). The

females as a rule incubated consistently for the remainder of the period after the male was removed. The separated male, frequently found in the corner of his cage nearest the brooding female, reacted to the movements of the female. Whenever she abandoned the nest, either to feed or to defecate, the male made evident attempts to get into the other pen, as if to occupy the nest. The resumption of the nest by the female quieted the male immediately.

A record of Experiment 2, in which females were separated, is given in Table II. These results differ from those of Experiment 1 in

TABLE I

EXPERIMENT 1

Showing the effect on the crop growth at the end of normal incubation period in males separated from, but left in sight of, their mates, after having incubated eggs for varying numbers of days

No. of Male	Stage of Incubation When Separated (Days)	Period of Separation (Days)	Stage of Incubation When Crop Examined (Days)	Condition of Crop
2192E	12	7	19	Normal growth
2348C	10	8	18	Normal growth
2300S	7	13	20	Normal growth
2348A	5	14	19	Normal growth
2281S	5	14	19	Normal growth
2317.1	3	16	19	Normal growth
2283R	3	16	19	Normal growth

lack of uniformity. In females 2326T, 2036A, and 2378A the changes in the crop were normal, while they were incomplete and variable in the rest of the females. In 2274R the condition was similar to that normally found at the twelfth or thirteenth day of incubation. The crops of 2326C, 2327G, 2273D, 2433B, and 2370E indicated little or no change.

After the isolation of 2326T, her mate occupied the nest for the rest of the incubation period. On the fourth day after separation the male did not attend very closely to incubation, but resumed with full intensity when the eggs hatched the next day. The behavior of this female while separated resembled that of the males in Experiment 1. In the case of 2036A and 2378A the mate continued sitting

on the eggs till the end of the normal incubation period, and the behavior of these females approximated that of 2326T. The eggs were neglected by the males in the rest of the cases before the normal incubation period terminated. The mates of 2274R, 2326C, 2327G, 2273D, 2433B, and 2370E continued brooding for 2, 2, 4, 1, 3, and 2 days, respectively, after these females were isolated. Abandonment of the nest by the male induced a change in the behavior of the fe-

TABLE II

EXPERIMENT 2

Showing the effect on the crop growth at the end of normal incubation period in females separated from, but left in sight of, their mates, after having incubated eggs for varying numbers of days

No. of Female	Stage of Incubation When Separated (Days)	Period of Separation (Days)	Stage of Incubation When Crop Examined (Days)	Remarks
2326T	13	6	19	Normal growth
2036A	12	7	19	Normal growth
2378A	12	6	18	Normal growth
2274R	13	6	19	Incomplete growth
2326C	10	9	19	Incomplete growth
2327G	10	9	19	Incomplete growth
2273D	10	9	19	Incomplete growth
2433B	9	9	18	Incomplete growth
2370E	8	10	18	Incomplete growth

male. She showed lack of interest in the activities of her mate and reverted completely to the non-brooding behavior.

The results of Experiment 1 alone might be taken to indicate that once the development of the crop is initiated, actual incubation, at least for a period longer than 3 days, is not necessary for full histological development and production of milk. In the case of the females, however, the development of the crop was incomplete if the birds were removed before 12 or 13 days of incubation (Expt. 2). This difference may reasonably be attributed to the fact that the mates of the females deserted the nest within 2 or 3 days after removal of the females, whereas, when the males were removed, the females continued incubation for the full period.

EXPERIMENT 3: SEPARATION OF MALE TO ANOTHER ROOM

In this experiment the males were placed in pens in another room, where they were unable to see their mates. Table III records the results, which show variability proportional to the elapsed time of incubation when the male was separated. Even in the males 2509B, 1953E, and 2345H, isolated as late as the twelfth day of incubation, the crop development was far from complete. The epithelium was thrown into folds, but there was no increase in its thickness. This is

TABLE III

EXPERIMENT 3

Showing the effect on the crop growth at the end of the normal incubation period in males separated from the sight of the brooding females, after having incubated eggs for varying numbers of days

No. of Male	Stage of Incubation When Separated (Days)	Period of Separation (Days)	Stage of Incubation When Crop Examined (Days)	Remarks
2509B	12	7	19	Slight change
1953E	12	7	19	Slight change
2345H	12	7	19	Slight change
2345F	10	9	19	Slight change
2274P	10	9	19	Slight change
2329H	3	16	19	No change
2348A	3	16	19	No change

comparable to the condition on the eighth or ninth day of normal brooding (Plate I, Fig. 2).

The separated males were unusually quiet for 2 days, following which their behavior reverted to the normal non-brooding type. There was no uniformity in the behavior of the females left with the eggs. Incubation was completed by the mates of 2509B, 2345H, and 2329H, while those of 1953E, 2345F, 2274P, and 2348A occupied the nest for 2, 5, 3, and 6 days, respectively, after the removal of their mates.

Kaufman (1932) reports an experiment apparently in conformity with the foregoing results.

In Experiments 1 and 2 the behavior of the separated birds of either sex resembled in some respects that of normal incubation.

Apparently, the act of the mate leaving the nest stimulated the separated bird in the same manner as when the two were together, and it may accordingly be argued that it was therefore in a similar psychological state. For convenience the behavior of the separated bird may be referred to as "psychological brooding," which persists only so long as its mate remains faithful to the nest.

EXPERIMENT 4: SEPARATION OF MALE TO ADJACENT PEN BUT WITHOUT VIEW OF MATE

The isolated males in this experiment were kept in pens adjacent to the brooding females as in Experiment 1; but a partition of thick cardboard, which eliminated the possible influence of sight, was placed between the birds. Five males were separated after respective incubation periods of 2, 4, 4, 3, and 6 days.

No change in the crops of these males was manifest at the end of the normal incubation periods. The behavior of the separated males, after 2 days of isolation, was similar to that in Experiment 3. The behavior of the females was again variable. Two occupied the nest till the end of the normal incubation period, while the others deserted their eggs on the second, fifth, and seventh day of separation, respectively. These results confirm those of Experiment 3, that sight of the brooding mate is involved in "psychological brooding."

EXPERIMENT 5: EFFECT OF STRANGE FEMALE ON MALE

Whether or not the influence of the sight of the brooding mate on the isolated bird can be inhibited in any way was tested by employing a strange female as a disturbing influence, for it was thought that this might lead the male to court and mate with the new female. The male was isolated as in Experiment 1, with only wire netting between; but a female, stranger to the male, was put with him. Three males were treated in this way, with varying results. The first, separated on the fourth day of incubation, paid no attention to the strange female. The crop hypertrophy was less than normal, the histological picture being similar to that of the twelfth or thirteenth day of normal incubation. The brooding female occupied the nest till the end of the incubation period. The second male was isolated on the third day of incubation. The new female introduced was aggressive, and frequently she and the male were observed fighting.

This did not, however, affect the incubating female, which continued normal incubation. The crop of the male showed no hypertrophy at the eighteenth day. The third male was separated on the fourth day of incubation and the next day was observed with the new female sitting on a nest provided for them. Two days later this female laid, but both eggs became broken; following this the male was observed frequently driving the female. At the time of completion of normal incubation by the original female the wall of the crop of the male was thin and transparent, like that of a non-brooding bird.

From the results of this experiment it is obvious that association with a new female inhibits, at least partially, the changes in the crop of the isolated male. It seems probable that the reaction of the male to the new female interrupts his state of "psychological" broodiness and hence stops the course of crop development.

EXPERIMENT 6: SEPARATION OF MALE FOR VARIOUS INTERVALS

The normal incubation period in pigeons, as has been mentioned, is about 18 days, or approximately 432 hours. The male normally devotes about one-fourth of this time, say 108 hours, to sitting on the eggs; and the female sits the remaining 324 hours. This may vary considerably under different circumstances. That the number of hours spent in actual brooding is not in itself the determining factor in crop hypertrophy is indicated by the fact that the development is complete in both sexes by the time the eggs are due to hatch. In the three cases now to be discussed the males were isolated as in Experiment 3 but were put back with their mates at intervals for short periods of varying length. As previously noted, when the males are removed early, the females tend to desert the nest. At such times it was necessary to return the males in order to have incubation continued. These periods of removal were found to have a rather definite effect on the male, depending primarily on the length of the periods during which he was isolated. Several trials were made, but only those carried to the end of the normal incubation period will be considered.

In Case A the first egg was laid on September 20 and the birds were examined on October 8, after 18 days of incubation, when the crops of both birds contained milk and showed some degree of hyper-

trophy. During the incubation period the total time that the male was removed from the nesting-pen, not counting a short interval on September 28, was approximately 207 hours in four intervals of 47, 48, 64, and 48 hours. His separation for this time did not modify the normal crop development.

The length of the period that the male was not with the female and the length of the intervals of isolation were increased in Case B. The time was cut down by 319 hours, in three periods of 111, 112, and 96 hours. This altered markedly the normal course of the changes in the crop of the male. At the end of the normal incubation period a piece of the crop was removed for histological examination. This showed the epithelium thrown into folds and slight hypertrophy (Plate II, Fig. 8). The capillaries from the vessels of the tunica propria had penetrated and entangled the cells of the stratum germinativum. These changes approximate those normally observed on the tenth or eleventh day of incubation. The reduction of incubation during the 18-day period in this case definitely retarded the normal changes. This raised the question whether crop development in the male could be continued by prolonging incubation to compensate for the reduced period. Infertile eggs were accordingly substituted before the original eggs hatched, and the birds were allowed to continue incubation as long as they would, the male now being left in all the time. By this means the incubation was extended over a period of 28 days—fully 10 days beyond the normal. After 150 hours the crops of both birds were again examined, and a piece from the male fixed for histological examination. This showed increased development, with the folds enlarged and the epithelium markedly thickened, but no milk (Plate II, Fig. 9). The histological picture resembles that of the thirteenth or fourteenth day of normal incubation (Plate I, Fig. 3). Complete crop growth and formation of the milk were, however, found when the male was killed and examined after an additional 96 hours. The epithelium was then enormously hypertrophied, and desquamation apparent (Plate II, Fig. 10). The extension of the brooding period by 246 hours thus corrected the retarding effect of the 319-hour reduction.

Case C repeated the experimental procedure of Case B. The normal incubation period for the male was shortened by 224 hours in

three intervals of 64, 104, and 56 hours. As a result of this decrease the changes in the crop of the male were not complete on the eighteenth day. There was a well-marked hypertrophy of the epithelium comparable to that obtained on the twelfth or the thirteenth day of

TABLE IV

EXPERIMENT 6

Showing effect of prolonged incubation following interruption in the case of the male

Case		17–18 Days		20 Days		22 Days		24 Days		28 Days	
		Brooding Time* (Hours)	Crop Development	Brooding Time (Hours)	Crop Development	Brooding Time (Hours)	Crop Development	Brooding Time (Hours)	Crop Development	Brooding Time (Hours)	Crop Development
A	♂	225	Normal hypertrophy *Milk*								
	♀	432	Normal hypertrophy *Milk*								
B	♂	113	Slight hypertrophy No milk					263	Normal hypertrophy No milk	359	Normal hypertrophy *Milk*
	♀	432	Normal hypertrophy *Milk*					582	Normal hypertrophy *Milk*	678	Not incubating
C	♂	208	Marked hypertrophy No milk	280	Increased hypertrophy No milk	328	Normal hypertrophy *Milk*				
	♀	408	Normal hypertrophy *Milk*	480	Not examined	528	Normal hypertrophy *Milk*				

* This indicates the number of hours the bird was in the cage with nest, not actual time sitting on nest. This time was divided between the female and the male when the latter was not separated.

normal incubation. Normal development was induced, as in Case B, by extending the incubation period by an additional 120 hours.

The results obtained in Experiment 6 are summarized in Table IV. In Case A the length of the separation periods ranged from 47 to 64 hours, and yet the crop changes were complete at the end of the normal incubation period. There is no evidence to show just how long after separation regression of crop development commences, but it is evident that, in this case at any rate, such changes had not gone

far enough to retard appreciably the time of milk formation. When the intervals exceeded 96 hours, as in Cases B and C, it was necessary by way of compensation to prolong the incubation beyond 18 days in order to secure the complete crop development. The most important fact disclosed by this experiment is that even during a single incubation it is possible several times to inhibit and to initiate again the changes in the crop by controlling the act of brooding, thus delaying the normal changes.

RELATION OF YOUNG TO THE FORMATION OF THE MILK

The fact that the formation of the milk coincides with the hatching of the young at the end of 17 or 18 days of brooding has led some investigators to speculate on the effects of the appearance of the young on the process. Thus Craig (1908) states: "In the very act of breaking the shell of the egg and emerging therefrom, the young dove begins to exert control over its parents; for the movements of the young excite the parents, confirm their attachment to the nest and its contents, and stimulate them to secrete the 'pigeon's milk' in the crop and to perform the feeding movements." At another place in the same paper this author further states: " . . . . ; after 14 days of brooding [he refers to doves], when the eggs hatch, both birds begin suddenly to secrete in the crop a food aptly called 'pigeon's milk'. . . . ." Whitman (1919, p. 66) observes: "The special secretions in the pigeon's crop seem to be stimulated in part by the sight of the young. . . . ."

A systematic study has been made to determine the effects of the young on the formation of the milk, (1) at the end of the normal incubation period, (2) before the end of the usual brooding period, and (3) during the feeding period.

AT THE END OF INCUBATION

To determine the effects of the presence or absence of young on the formation of the milk at the end of the incubation period, it was only necessary to have birds incubate infertile eggs. This opportunity was afforded in the castration experiments, where the birds incubated the infertile eggs. At the end of 18 days of brooding, the crops of all the females contained milk in spite of the fact that there were no young to excite the formation of the milk. It is evident,

therefore, that the formation of the milk proceeds even in the absence of the young.

BEFORE THE END OF INCUBATION

Several tests were made by giving squabs from other parents to birds at different stages of incubation.

The evidence from these experiments is conclusive that the process of the formation of milk can be hastened very little, if at all, by the premature presence of young in the nest. These results corroborate the statement of Fulton (1895, p. 43), who observes on this point:

> We have seen how easily, in the case of most pigeons, the young ones may be "shifted" at almost any time desired within a fortnight; and pigeons will also readily take to and sit upon other eggs than their own; but it will *not* answer to give to any pair eggs partly hatched, unless laid at the same time as their own, and therefore due to hatch at the same date. The reason is obvious; the eggs hatching before the ordinary time of incubation is expired, there is *no soft food ready* for the young, and they must therefore perish. One day or perhaps two does not matter; but success when the shifted eggs have been sat upon more than this is very doubtful.

DURING THE FEEDING PERIOD

The young are fed milk alone for the first 2 or 3 days. As they grow older, the milk is replaced partly with grain; after they are about 7 or 8 days old, grains are fed exclusively. If the feeding of milk alone for the first 2 or 3 days is a response to the age of the young, it should be possible to prolong the period of formation of the milk by substituting newly hatched squabs at regular intervals. The validity of this assumption was tested in several experiments, the results of which were negative in all cases.

Some light is thrown on the problem of the exclusion of grain from milk fed the young for the first 2 or 3 days by observations made on the condition of the crops of the parent birds during incubation and feeding of the young. From the seventeenth day of incubation to the fourth day of feeding, grain is not found in the crops of the parent birds, even just after they are fed. This is apparently due to the enormous hypertrophy of the epithelium, which greatly decreases the cavities of the pouches. The space which remains is occupied by milk, and thus no place is available for the storage of grain. From the fourth day of feeding onward, the processes of cell multi-

plication and desquamation begin to regress, and consequently more space is available for the collection of grain. It is apparent, therefore, that the omission of grain for the first 2 or 3 days and its subsequent inclusion in the feed of the young is not due to any special regulatory power possessed by the pigeons but is the direct result of the histological changes in the crop during this period.

RELATION OF TESTES TO MILK FORMATION AND TO INCUBATION

CASTRATION

Twenty-one males were castrated, some with complete success; in other cases the castration was incomplete. All but two of these males were already mated at the time of castration. The castrate period before the beginning of incubation was partly controlled by breaking up the reproductive cycle at the start of a previous brooding. Two males were castrated in the beginning of their first incubation.

GROUP 1: CASTRATION COMPLETE

Effects on the changes in the crop.—In completely castrated males normal crop development was found at the end of the first incubation regardless of whether the operation was performed during the incubation period or at some time previous to it. The castrate periods before the commencement of the first incubation ranged from 3 to 16 days.

During the second and subsequent incubations after the operation the effects of the castration were evident. In all of these birds the histological picture of the crop was indistinguishable from that in Plate I, Figure 1, which represents the non-brooding stage. The castrate period before the beginning of the second incubation varied from 28 to 78 days.

Effects on behavior.—Changes in sexual behavior caused by prepuberal and postpuberal castration in other birds have been reviewed by Lipschütz (1924); and, as was to be expected, the results in the pigeon showed a more or less complete suppression of sexual activities. Incubation behavior did not disappear completely in all the castrated males, although there was a progressive decrease in its intensity and regularity. In the incubation period during which the

operation was performed, the brooding behavior and crop development remained normal; the male showed the same degree of stubbornness in leaving the nest as does an unmutilated male. During subsequent incubations, however, a mere approach to the pen sufficed to make the male leave the nest, and the crop changes did not occur.

Two males were unmated at the time of castration. Five weeks after the operation they were put with females but showed no interest in them and lacked the pugnacity so obvious in normal males placed with strange birds. The courtship behavior was entirely lacking. Nevertheless, in spite of the lack of courtship and mating, the females with these castrated males laid eggs several times. Kaufman (1932) obtained results consistent with those here reported.

The fact that the male would continue incubation, produce the milk, and feed the young after being castrated early in the period was also demonstrated by Riddle and Dykshorn (1932); but these investigators did not carry the experiment beyond the one period.

GROUP 2: CASTRATION INCOMPLETE

Despite the fact that the amount of testicular tissue left in some males was exceedingly small, such males exhibited masculine behavior during courtship and normal crop development during incubations. No intermediate condition either in the growth of the crop or in behavior was noticed. In one case the testicular tissue found at autopsy weighed only 0.025 gm., which is about 1.4 per cent of the total testicular tissue in a normal male. Active spermatogenesis was the rule in the testicular nodules. There was, however, no appreciable increase in the amount of intertubular tissue.

Kaufman (1932) also mentions normal nesting behavior and crop development in an incompletely castrated male pigeon.

MALE HORMONE

It seems apparent that the effects of castration, which prevents crop hypertrophy in the male during the second and subsequent incubations following the operation, are due, at least indirectly, to the absence of a testicular incretion. Attempts were accordingly made to rebuild the normal condition by injections of preparations of male hormone. The urine of young men was used, the method of extrac-

tion being that of Funk, Harrow, and Lejwa (1929, 1930). The potency of the extracts was tested by their effects on comb growth in capons.

Five castrated males were injected intraperitoneally three times a day with 0.5 cc. of urine extract (1 cc. = 200 cc. of urine) from the first day of incubation to the end of the incubation period subsequent to the first, but no crop development resulted. Consequently four males were injected three times a day with 0.5 cc. of a more concentrated extract (1 cc. = 400 cc. of urine). These results were also negative. The "dosages" employed varied from 3 to 6 cock units a day, which should eliminate the question of insufficiency of the hormone as an explanation for the negative results.

Two castrated males were injected intraperitoneally every other day during the incubation period with 1 cc. of blood serum from normal brooding males. The results were negative in both the birds.

In another experiment six testes from normal incubating males were implanted in two castrated males during the incubation periods of the latter. No positive effects were produced during the incubation in which the implantations were made, nor in the one following. At autopsy the implants were found to be resorbed.

Kaufman (1932) employed aqueous extract of pigeon testes on both brooding and non-brooding castrate males, also with negative results.

CHANGES IN THE TESTES DURING INCUBATION

A correlation between the activity of the crop during incubation and the gonads was reported by Champy and Colle (1919). They report reduction in size of the testes during incubation reaching a maximum just before the hatching of the young, after which there is a gradual return to normal. Accompanying the reduction in size they reported a histological regression consisting of resorption of a large number of tubules and suppression of spermatogenesis in the remaining tubules. These changes, however, were unlike those observed in the winter season, for during incubation the interstitial tissue was absent in the testes, while it is abundant during the winter. In the female, during incubation, they observed an atresia of a large number of oöcytes; and this, as in the male, attained its maximum at the end of the period, thus corresponding in time with the maximum changes in the crop.

Our observations on the testes during incubation do not agree with those of Champy and Colle. The average weight of testicular tissue in six non-brooding males was 1.825 gm., and in six incubating males 1.816 gm. The difference is not significant. We have found neither a reduction in the number of tubules nor a suppression of spermatogenesis during incubation. This is evident in Plate II, Figure 11, which is a photograph of a section of a testis on the sixteenth day of incubation. The different stages of spermatogenesis are well marked.

DISCUSSION

The experiments herein reported demonstrate clearly that the act of brooding has a definite relation to the normal development and functioning of the crop in the production of "milk." What part the sex glands play in the process is not quite so clear. Riddle and Braucher (1931) first showed that by the use of anterior pituitary extracts the crop development could be induced in male pigeons that had been castrated several months before. Later it was determined (Riddle, Bates, and Dykshorn, 1932, 1933) that the principle concerned is distinct from the already-known growth and sex-maturity hormones of the anterior pituitary, and it was accordingly designated "prolactin." Riddle and Dykshorn (1932) summarize their conclusions regarding the rôle of the testes as follows:

> There is no valid evidence that the testes play any direct part in the activation of the crop-glands. The testes do, of course, induce or influence the copulations which normally lead to the male's impulse to incubate; and a completed term of incubation apparently induces the anterior pituitary to release the specific hormone which in turn activates the crop-glands. The presence of this activator in the blood is as effective in the absence as in the presence of the testes.

Our results, as well as those of Kaufman, have shown that castrated males do not have the crop development in incubation periods beyond that following the operation, even though they may continue to incubate in relatively normal fashion; and it is to be presumed that the pituitary in this case is not producing the specific hormone for crop development. Riddle and Dykshorn are inclined to attribute this result, in a case earlier reported by Kaufman and Dabrowska (1931), to a lack of synchrony with the mate or to less intense incubation on the part of the castrated male. While this may

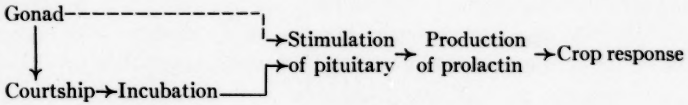
have been true in that particular case, there is room for considerable doubt as to whether it would apply to our cases or to the later ones reported by Kaufman (1932). Unfortunately, our records are not sufficiently complete to allow a positive statement as to the intensity of incubation by the castrated males that remained continuously with their mates—critical evidence would require almost continuous observation; but on the basis of observations made two or three times a day and oftener, they appeared to be carrying their regular share of the responsibility. The experiments on the temporary periodic removal of normal males demonstrated that the crop development is closely dependent on the length and continuity of incubation; but it is still possible that a more intense initial stimulus—such as copulation—is necessary to initiate the pituitary activity, and we have no evidence that this occurs in the case of the castrated males.

Another interpretation seems possible and has some evidence in its support. That the testis is necessary for males not yet mated to pair up is indicated by the behavior of two birds castrated before being mated, and which thereafter, though tried with females, would not mate. On the other hand, when males of mated pairs were castrated, they continued to respond to the incubation cycles of their mates even though the operation was 12–16 days prior to laying of the eggs. In these males, furthermore, the crop glands functioned in the first incubation period following the operation, but not thereafter. The difference between the first and subsequent incubation periods after operation may logically be attributed to the persistence of testicular influence for a period following the operation. Whether this influence is the direct action of a sex hormone, or whether previous mating has conditioned the nervous system so that mating behavior continues, cannot be decided on the basis of present facts. It would appear, however, that the production of prolactin by the anterior pituitary depends not alone on the psychic stimulation, but that there must either be sex hormone present at the time or the pituitary must previously have been conditioned by such a hormone. It is to be remembered that the prolactin used by Riddle and his associates to induce crop development in castrated pigeons came from pituitaries of animals (mammals) which presumably had developed it under the influence of active sex glands, at least of ovaries.

It would be interesting to determine whether the pituitaries of *castrated* mammals only would produce the same reaction.

The fact that the crop milk is formed by female as well as by male pigeons demonstrates that, whatever influence the sex gland has, it is not peculiar to the testis. This does not, however, rule out the possibility that the same hormone may be operating in the two sexes, since Kabak (1933) reports finding male hormone present in the urine of women in sufficient amounts to produce the typical comb responses in castrated cocks.

The alternative suggestions as to the chain of events leading to the crop hypertrophy here presented differ only in the rôle attributed to the gonad. This may be illustrated by the accompanying diagram:



The simple chain of events, as suggested by Riddle, would be represented by the diagram if the broken-line arrow is omitted. The gonad stimulates courtship, which is followed by incubation; this chain stimulates the pituitary to the production of prolactin, followed by crop enlargement. This view encounters the difficulty of explaining the failure of incubation to induce the pituitary action in castrated males which continue incubation through several cycles. The other suggestion is that the gonad is not only necessary for initiating normal sex behavior but it also influences the pituitary directly (or possibly by previous conditioning) to produce the crop-developing hormone. In connection with this view, however, it must be recalled that attempts to supply the male hormone in incubating castrated males did not lead to the crop response. Further investigation will be required to determine the relative rôles of the sex glands and of the psychosexual behavior.

SUMMARY

1. The histological changes in the crop during incubation and feeding of the young were studied in closely graded stages. The observations confirm those of Litwer (1926) and Beams and Meyer

(1931) that the "pigeon's milk" consists of masses of the epithelial cells, loaded with fat globules, from the wall of the lateral pouches of the crop of the parent birds. The histological changes leading to the formation of the milk are gradual and consist in an increased cell multiplication in the stratum germinativum, followed by desquamation of the surface cells of the thickened epithelium.

2. If the male of a mated pair is put in a separate cage adjacent to and whence he can see his incubating mate, his crop has the normal development at the end of 18 days, and he is capable of feeding the young. This influence, in the absence of actual brooding, is referred to as "psychological brooding."

3. Removal of the male from sight of the female, whether close by or in another room, prevents the progress of changes in his crop. Under these conditions the psychological brooding does not occur.

4. The changes in the crop of the male can be checked and started again several times during the same incubation period by alternating the normal brooding with periods during which he is prevented from sitting on the eggs and is also removed from sight of the incubating mate.

5. As a result of the long intervals of the lack of both actual and psychological brooding, interrupted by the short periods of normal brooding, the changes in the crop of the male do not attain their normal condition within the usual incubation period. Normal crop development may, however, be secured by prolonging the incubation beyond 18 days.

6. Formation of milk occurs at the end of 18 days of incubation even in the absence of squabs.

7. The substitution of young for eggs early in the incubation period does not provoke the formation of milk before the normal time.

8. It was found impossible to prolong the period of milk formation by substituting newly hatched squabs successively during the feeding period.

9. The exclusion of grain from the food of the young for the first 2 or 3 days is attributed to the great thickening of the crop of the parents, which leaves no space for the storage of grain.

10. When young do not supervene at the normal hatching time, milk formation continues as long as the birds incubate the eggs beyond 18 days.

11. Castrated males that were mated at the time of castration produce "milk" normally during the first following incubation period but not in succeeding ones.

12. If castration is incomplete, crop hypertrophy is normal in all succeeding incubations even though the piece of testicular material remaining is very minute.

13. Extract of male urine, blood serum from brooding male birds, and implants of testis into inactive castrates failed to produce the crop response.

14. The observation of Champy and Colle that there is an involution and decrease in size of the testis coincident with the increasing hypertrophy of the crop was not confirmed.

15. While it is possible that the gonads influence milk formation only indirectly through their effect on sexual behavior, it is still an open question whether they may not also have a direct effect on the pituitary activity.

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THE OXYGEN CONSUMPTION OF HEXAGENIA RECURVATA DURING THE WINTER AND EARLY SPRING

(Three figures)

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LIKE other May flies, the large burrower, *Hexagenia recurvata*, lives in the water during its immature or nymphal stage, which in this species lasts for about two years. These nymphs live in ponds and slow streams, tunneling their way through the mud or creeping over the bottoms. Where they occur at all, they are usually abundant and obtainable throughout the winter in goodly numbers. The nymphs used in these experiments were taken from one region of a spring-fed brook near South Hadley, Massachusetts, where *H. recurvata* nymphs were also collected for an earlier study of the functions of their gills (Morgan and Grierson, 1932).

MATERIAL AND METHODS

Material.—Eighteen collecting trips were made to Lithia Springs Brook during the fall and winter (September 19, 1934—March 15, 1935) and one trip on May 3. Observations of weather conditions, temperature of the water, and activities and abundance of the nymphs were made on each trip. Nineteen hundred and eighty-five nymphs, ranging from 10 to 30 mm. in length, were collected.

Immediately after the field trips the nymphs were measured and caged in groups according to size. Two kinds of cages were used. The stock cages were 1 foot in diameter and had sides and bottom of copper-wire screen. These were set in galvanized iron trays 2 inches deep, each having a capacity of 150 nymphs. The small cages holding from one to seven nymphs were used for those which had been chosen for observation. Each of these was a 3×4-inch compartment separated off by wire screening in a multiple-unit tray of forty such cages. All cages were supplied with running water and diatom-saturated ooze from the nymphs' own habitat. Fresh mud was

added from time to time, and in February filamentous algae were stirred in with it.

About once a week the small cages were examined for molted skins and dead nymphs. The stock cages were examined once a month. Four other cylindrical screen cages, containing a total of about twenty nymphs, were kept in Lithia Springs Brook as a check on the condition of the laboratory-kept nymphs.

During the spring and fall the death-rate was high. Through the winter, however, probably as a result of the lowered metabolism accompanying winter conditions, the death-rate was lowered to about one out of eight nymphs per week.

Organization of the experiments.—Twelve experiments were conducted from October 27 to March 17, but the first three of these were discarded because of faults in technique. Of the remaining nine, three were performed in December (December 1, 15, 29), three in January (January 2, 12, 29), two in February (February 9, 23), and one in March (March 16).

Each experiment was set up as follows: three 500-cc. bottles each containing 250 cc. of distilled water and three nymphs and one bottle with the same water content but no nymphs (the blank) were set in each of three water baths, whose average temperature was respectively 1.0°, 6.4°, and 12.3° C. Thus, twelve bottles were used. Of the nine containing nymphs, usually two out of the three in each temperature held recently collected nymphs, while the others held nymphs that had been kept in the laboratory from 10 days to 2 months.

The experiments lasted 25 hours. Five determinations of the oxygen content of the water in the bottles were made, namely, at the start and at the fifth, tenth, fifteenth, and twenty-fifth hours.

Care of nymphs before the experiments.—After the nymphs had been brought to the laboratory, they were at once sorted and grouped according to size. Those which were to be kept in the laboratory were placed either in the stock or unit cages before described. The largest nymphs available (approximately 27 mm.) were used in the experiments. Sometimes these could not be secured and it was necessary to use 24–25-mm. lengths. They were acclimated by being placed in bottles set in water baths maintained at the temperatures

at which the tests were to be made. It was hoped that the period of acclimatization would provide time for the digestion of food already consumed, as well as accustom the nymphs to the temperature at which they were to be tested. The 500-cc. bottles used for acclimatization were the same ones in which the nymphs were later tested. Wire mesh was placed in the bottom of each bottle as a foothold for the nymphs.

Maintenance of constant temperature.—The lowest temperature, 1° C. used in the experiments was obtained by the use of an electrically controlled constant-temperature bath. The medium temperature was maintained by an even flow of water into a museum jar containing the experimental bottles. This temperature remained constant ±1° through an experiment, but varied from month to month, showing a slow decline from 7.0° C. on December 1 to 5° C. on March 15, with a seasonal average of 6.4° C. The highest average temperature, 12.3° C. (average of the highest temperatures in each of nine experiments) was obtained by running tap water through a 1-liter Erlenmeyer flask heated by the coils of an electric unit. After some experimentation this temperature could be kept constant ±3° during an experiment, but it varied slightly from experiment to experiment.

Oxygen determinations.—Following the practice of Hiestand (1931), during experimentation all nymphs were kept in distilled water, which appeared to have no harmful effect upon them. Since the determinations were made by the unmodified Winkler method, it was thought that by the use of the distilled water errors due to impurities in the water could be avoided. The distilled water was brought to the right temperature and, just before using, was shaken up with air in order to oxygenate it.

The experiments were started in the morning following a collection of the nymphs. Bottle A, containing nymphs collected the day before and acclimated at 1.0° C. through the night, was taken from the water bath. The acclimatization water was poured out. The bottle was then rinsed with distilled water of the same temperature, and finally 250 cc. of distilled water was siphoned into the bottle. A deep layer of motor oil was then poured onto the water. The cork was pressed in and oil added through a small hole in the cork till the

bottle was completely full. The hole was then plugged, and the whole top coated with melted paraffin. The bottle was then completely submerged in the water bath. In the earlier experiments the glass bend of the exit siphon was left above water, but bubbles of gas collected there; so in later experiments it was submerged. Fifteen minutes after the procedure with bottle A, the same thing was repeated for bottle B; 15 minutes after that, for C; and so on. Thus, each of the twelve bottles was started in sequence 15 minutes apart. The determinations were made at the fifth, tenth, fifteenth, and twenty-fifth hours and each took 15 minutes to perform; thus the contents of each bottle could "run" the same length of time. Before each experiment determinations were made of the oxygen content of the water to be used in the test bottles.

The determinations were made in a Thompson-Miller apparatus for the micro-Winkler method, 5.5 cc. capacity. Before each test this was filled to capacity with water from the experimental bottle, then emptied, and refilled to capacity for the test. Thus, equal amounts of water were always removed from all bottles in each series of determinations. After each test the apparatus was rinsed, first with tap water and then with distilled water. Directions for the use of the apparatus are given by Thompson and Miller (1928); and notes on its accuracy, by Snoke (1929) and Allee and Oesting (1934). As soon as the sample had been drawn from the bottle, more oil was added in order to bring the oil surface to the level of the cork again. The top was then resealed with paraffin. The condition of the nymphs was always noted during these operations and before the bottle was replaced in the bath.

The water sample was treated according to the method of Kremmerer, Bovard, and Boorman (1923) and modified to suit the Thompson-Miller micro-apparatus. The sodium thiosulphate was standardized against N/100 potassium permanganate instead of against potassium bichromate. The equation used was that given by these workers for finding the amount of oxygen in cubic centimeters per liter. This was changed to milligrams, and the milligrams per gram body weight computed.

The use of motor oil as a seal.—Motor oil was used to cover the water during the tests in all the experiments. It was finally adopted

after considerable experimentation and practice. In preliminary experiments the water was covered with paraffin oil (Amberson, Mayerson, and Scott, 1924; Morgan and O'Neil, 1931; and others). It is well known that atmospheric oxygen will penetrate through paraffin oil, especially after periods longer than 10 hours. These experiments on *Hexagenia* lasted 25 hours. The 500-cc. bottles were chosen in order to allow for a deep layer of paraffin oil over the water. Even with this precaution some leakage was detected by a rise of oxygen in the water of the blank bottles.

By the use of luminescent bacteria Hill (1928) showed that oxygen would go through paraffin oil and into water within 6 hours but that it would not go through motor oil. In the practice experiments with the motor oil it was discovered that with this oil oxygen passed from the water into the oil. There was an apparently greater oxygen consumption by the nymphs in water covered by motor oil than there had been by those in water covered by paraffin oil, and the oxygen content of the blank bottles fell in proportion to the temperature. No deduction for the error inherent in the motor oil was made in the calculations of the oxygen consumption. However, the average loss from the water into the oil at three temperatures was measured and is presented in Table I.

In other experiments in this laboratory paraffin oil has served as well as motor oil when the test bottles were totally submerged and duplicate determinations averaged. Either motor or paraffin oil gave only comparative results. These, however, are satisfactory for the biological questions at hand. For absolute measurements, bottles should be sealed with mercury or inclosed in a nitrogen chamber.

OBSERVATIONS

At a given temperature the average oxygen consumption of *Hexagenia* nymphs was nearly the same through the four succeeding 5-hour intervals from one determination to another (Table II). But variation in the rate of consumption occurred in individual sets of animals. Such variation of individual oxygen consumption from hour to hour is not uncommon amongst invertebrates (Dakin and Dakin, 1925, p. 315).

At the lower temperatures 1.0° C. and 6.4° C. the consumption of the nymphs was unaffected by the decreasing oxygen tension. At

TABLE I

AMOUNTS OF OXYGEN THAT ESCAPED FROM DISTILLED WATER INTO A SEAL OF MOTOR OIL IN EXPOSURES MADE AT THREE TEMPERATURES

Amounts given are the averages of determinations made on the "blank bottles" of five experiments (January 1 through March 16), given in milligrams of oxygen per liter of water.

Temperature Degrees C.	Oxygen Content at Start	Oxygen Loss at—			
		5 Hours	10 Hours	15 Hours	25 Hours
1.0	9.603	0.552	0.737	1.313	2.189
5.64	8.619	0.476	0.745	1.465	2.356
11.88	7.491	1.349	2.158	2.856	4.130

TABLE II

THE COMPARATIVE OXYGEN CONSUMPTION OF RECENTLY COLLECTED (REC. COL.) AND LABORATORY-KEPT (LAB. KEPT) NYMPHS OF *Hexagenia recurvata* DURING 5, 10, 15, AND 25 HOURS AT THREE TEMPERATURES

Average of twenty-fifth hour determinations at 12.3° C. based on January–March only. An average of nine experiments. Amounts of oxygen in milligrams per gram body weight.

Hours of Consumption	Nymphs	1.0° C.	6.4° C.	12.3° C
5	Rec. col.	2.305	2.982	5.510
	Lab. kept	1.444	2.503	4.460
10	Rec. col.	3.582	6.058	9.387
	Lab. kept	1.960	4.592	8.229
15	Rec. col.	5.006	8.598	12.174
	Lab. kept	3.359	6.885	11.558
25	Rec. col.	7.584	11.950	8.66
	Lab. kept	5.203	10.692	7.89

12.3° C. the consumption decreased between the fifteenth and twenty-fifth hour, but this was probably due to the effect of accumulating carbon dioxide.

Singh-Pruthi (1927) found that May-fly nymphs could remain in water with oxygen tensions as low as 0.3–0.2 cc. per liter without ap-

parently being affected, provided the pH and the tension of carbon dioxide were right. Many *Hexagenia recurvata* nymphs died after 15 hours at 12.3° C., and it is believed that carbon dioxide narcosis may have been responsible.

The oxygen consumption of recently collected and laboratory-kept nymphs.—The average oxygen consumption of nymphs kept in the laboratory 10 days or more is less than that of nymphs collected within 24 hours.

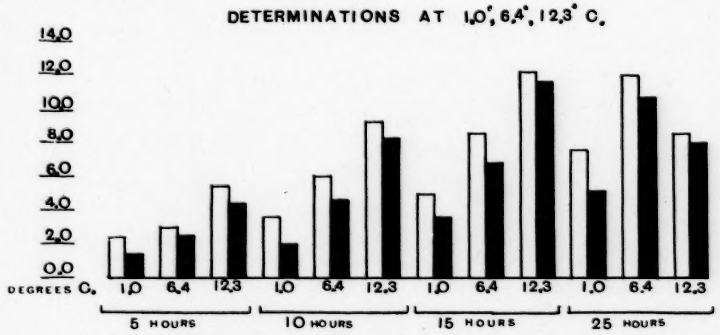

FIG. 1.—The comparative oxygen consumption of recently collected (white) and laboratory-kept (black) nymphs of *Hexagenia recurvata* at three temperatures for 5, 10, 15, and 25 hours. Averages of results of nine experiments are represented covering a period from December 1 through March 17. December–January determinations lacking for twenty-fifth hour at 12.3° C. (Fig. 2, 12.3° C.); hence, average for whole year reduced out of proportion to other columns. Ordinates, milligrams of oxygen per gram of body weight.

Averages of the results of nine experiments each involving an average of 22.8 nymphs revealed that laboratory-kept nymphs showed a consistently lower oxygen consumption than recently collected ones (Table II and Fig. 1).

The exception indicated in Figure 2 in the fifteenth-hour determinations (December 1—January 13) at 12.3° C. is only an apparent one. Several of the recently collected nymphs involved in these 12.3° C. tests showed a lessened resistance toward the end of the experiments, which allowed the laboratory-kept nymphs to overtake them in oxygen consumption. In several cases the nymphs died by the fifteenth hour. From December 1 to January 13 no consumption record was used for the twenty-fifth hour at 12.3° C., because one or

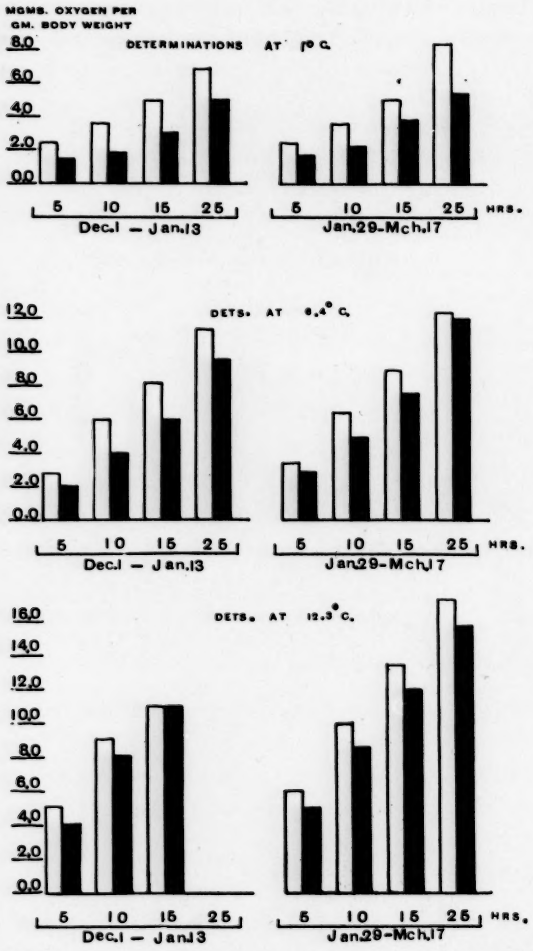

FIG. 2.—The average oxygen consumption of recently collected (white) and laboratory-kept (black) nymphs of *Hexagenia recurvata*, showing (1) the early-winter period of low oxygen consumption followed by the late-winter and spring rise, and (2) the higher consumption of the recently collected nymphs (for explanation of exception in fifteenth-hour determination at 12.3° C. see p. 159). Abscissa, intervals at which determinations were taken; ordinate, milligrams of oxygen per gram of body weight. Averages based on five experiments for the first and four for the second periods.

two out of the three nymphs were dead before this hour of this series. Such records were discounted (twenty-fifth hour, 12.3°, Table III).

TABLE III

THE AVERAGE OXYGEN CONSUMPTION OF RECENTLY COLLECTED AND LABORATORY-KEPT NYMPHS OF *Hexagenia recurvata* FOR THE EARLY-WINTER (DECEMBER 1—JANUARY 13) AND THE LATE-WINTER PERIOD (JANUARY 29—MARCH 17)

Tests made at three temperatures. Amounts of oxygen in milligrams per gram of body weight. Average based on nine experiments each involving an average of 22.8 nymphs.

Hours of Consumption	Season	1.0° C.	6.4° C.	12.3° C.
	Recently collected nymphs			
5	Dec.–Jan.	2.265	2.260	4.998
	Jan.–March	2.346	3.305	6.021
10	Dec.–Jan.	3.508	5.774	8.927
	Jan.–March	3.657	6.343	9.847
15	Dec.–Jan.	4.948	8.241	10.936
	Jan.–March	5.063	8.955	13.411
25	Dec.–Jan.	6.805	11.479	.....
	Jan.–March	8.362	12.420	17.327
	Laboratory-kept nymphs			
5	Dec.–Jan.	1.304	2.079	3.970
	Jan.–March	1.585	2.927	4.950
10	Dec.–Jan.	1.709	4.024	7.804
	Jan.–March	2.210	5.160	8.654
15	Dec.–Jan.	2.944	6.036	10.995
	Jan.–March	3.774	7.734	12.122
25	Dec.–Jan.	5.016	9.503	.....
	Jan.–March	5.390	11.801	15.784

Correlated with the higher metabolism indicated by the respiration of the recently collected nymphs was the fact that between March 15 and May 3, 78.4 per cent of them had molted, while between April 15 and April 29 only 5.3 per cent of the laboratory-kept nymphs had done so.

As evidenced by the oxygen consumption just mentioned, it appears that the metabolism of the nymphs was lowered by laboratory conditions, even though the temperature of the water in which they were kept was approximately 5° higher than that of their own habitat. This emphasizes the fact that in physiological studies it cannot be taken for granted that animals are living at their normal rate. Dreyer (1932) calls attention to the importance of the imitation of natural conditions for animals that are being used for physiological experiments. He was able to imitate the ant's normal habitat and found that the respiratory behavior of laboratory colonies was "similar to that of animals brought directly from the field nests." The laboratory conditions in which *Hexagenia recurvata* lived were certainly as good as those of most aquatic animals in the laboratory. These nymphs were in constantly flowing water of high oxygen content and a pH of 6.8, only two-tenths less than that of their own habitat, Lithia Springs Brook. Lack of space seems unlikely to have hurt them, since a group of fifteen nymphs tried out in a 3×4-inch cage apparently lived as well as did one to five nymphs in cages of the same size. Metabolic products could not have been an influence, since currents of water constantly cleared these away. However, there were obviously unavoidable differences between the laboratory and native environments, such as those incident to depth of water and to materials in solution.

Oxygen consumption as affected by season.—The oxygen consumption of all nymphs was higher in February and March than it had been earlier in the winter. When the consumptions in five early winter experiments (December 1 through January 3) were averaged and compared with the four late winter ones (January 29 through March 17), the results showed that the late ones were greater in every case (Table III and Fig. 2).

This seasonal rise involves the problem in much greater complexity. If the metabolism of the nymphs were dependent upon the temperature of the environment, as Dreyer (1932) has found for the ant, a constant temperature would lead one to expect a steadily maintained metabolism, and a falling temperature a lowered metabolism. But the rise in oxygen consumption was not correlated with a rise in habitat temperature in either freshly collected or in labora-

tory-kept nymphs, as records of habitat temperature and oxygen consumption show (Fig. 3, graphs *A*, *B*, and *C*). The January rise

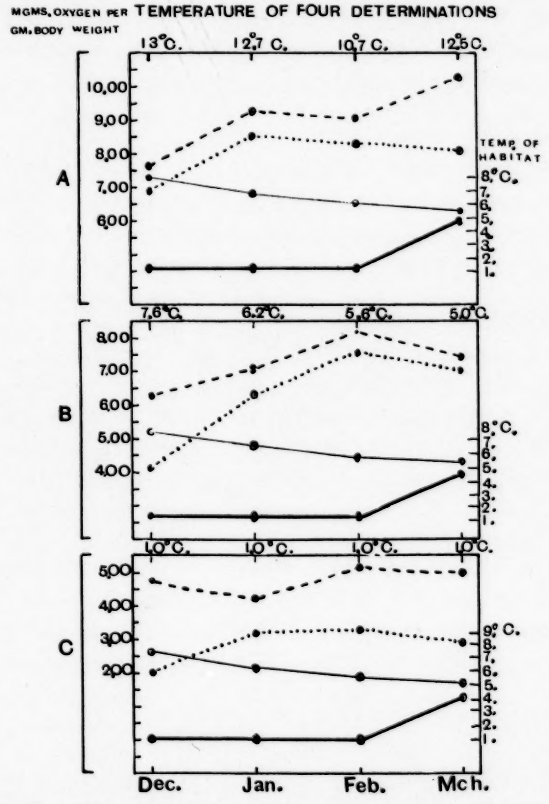

FIG. 3.—The average oxygen consumption of recently collected (heavy dash line) and laboratory-kept (light dot-and-dash line) nymphs of *Hexagenia recurvata*, December 1 through March 17, showing monthly fluctuations and the temperature of their habitats: temperature of native stream (heavy continuous line), temperature of tap water (light continuous line). Abscissa, months when determinations were made; ordinate, milligrams of oxygen per gram of body weight.

occurred, without exception, in both types of nymphs, except that in those tested at 1.0° C. it was not found until February (Fig. 3, graph *C*).

In the laboratory-kept nymphs and in the recently collected ones tested at a temperature higher than that at which they had been living the late-winter rise was first noticed in January (Fig. 3, graph *A*). In recently collected nymphs tested at the temperature of their habitat (1.0° C.) the rise occurred in February (Fig. 3, graph *C*). None of these rises can be correlated with temperature, since in Lithia Springs Brook the temperature was about the same as on earlier winter dates (December, 1.5°; January, 1°; February, 1° C.) and in water at the laboratory it was lower (Fig. 3, graphs *A*, *B*, *C*, temperature of habitat). A correlation of increased body weight and proportionately lowered oxygen consumption was observed in the March determinations.

The fall in the March consumption of recently collected nymphs shown in Figure 3, graph *B*, may be correlated with the fact that these nymphs were the heaviest ones collected. In individual experiments it was found that a heavy nymph consumed proportionately less oxygen than a lighter one. The converse is shown in graph *A* of the same figure, where the March rise is in the oxygen consumption of lighter-weight nymphs. At this season the greater growth activity of these smaller nymphs created a greater oxygen demand, in addition to that resulting from the greater proportion of surface to the volume of their bodies.

Contrary to these observations, Dreyer (1932), as already mentioned, found that the oxygen consumption and respiratory exchange in the ant paralleled the temperature, whether natural or experimental. He kept ants, *Formica ulkei*, in a hibernating condition throughout the year by placing them in a low temperature, and in an active condition by keeping them in a high temperature, and determined that their respiratory exchange closely followed their surrounding temperature throughout the year.

He has thus shown that the rate of metabolism in the ant is generally dependent upon environmental temperature. When the temperature was varied experimentally, the oxygen consumption of nymphs of *Hexagenia recurvata* was also affected by the temperature (Tables II and III; Fig. 1). On the other hand, in January and February their oxygen consumption rose in spite of consistently low temperatures (Fig. 3). Thus it becomes apparent that, while these

nymphs may be affected experimentally by temperature, their metabolism is nevertheless, to a considerable degree, independent of it. It is likely that there is a similar situation in the winter stone flies, as their winter behavior demonstrates. *Capnia* and *Taeniopteryx*, which are active, grow rapidly and even emerge into the air as adults as early as January and February when the temperature of their habitat is as low or lower than it has been earlier in the winter.

The oxygen consumption as affected by experimentally varied temperature.—In these experiments, as already stated, three temperatures (1.0°, 6.4°, and 12.3° C.) were used, and three levels of oxygen consumption resulted (Tables II and III, and Figs. 1 and 2). The objection may be raised that the absorption of oxygen by the motor oil might account for these levels. This objection has been considered. The loss of oxygen from the water into a seal of motor oil was determined for three temperatures at 5-, 10-, 15-, and 25-hour intervals (Table I). The loss of oxygen in the oil at 25 hours at 1.0° C. was, on the average, 2.189 mg. of oxygen per liter of water; at 5.6° C. it was 2.356 mg.; and at 11.88° C. it was 4.130 mg. The increase in loss of oxygen between 1.0° C. and 5.6° C. is not proportionate to the much greater loss, owing to its consumption by the nymphs (cf. Tables I and II). With the experimentally varied temperatures the average increase in oxygen consumption with a rise of 6° was 1.26 times that at 1.0° C.; and with a rise of 12°, 2.77 times that at 1.0° C. When these figures were calculated on the basis of the Q_{10} of van't Hoff (Rogers, 1929), a Q_{10} value of 3.319 was obtained from the temperature rise of 1.0°–6.4° C., and 2.645 from the rise of 6.4°–12.3° C. The smaller Q_{10} for the rise from 6.4° to 12.3° C. indicates that at the higher temperatures factors such as carbon dioxide accumulation (Fig. 2, December 1—January 13, 15-hour determination, and death of nymphs before 25 hours) were probably operating on the nymphs to reduce their metabolism.

DISCUSSION

The fact that *Hexagenia recurvata* consumed less oxygen during the first part of the winter indicates its depressed metabolism in that season. Possibly in November, certainly in December and the first

part of January, occurs its winter "low." Near the end of January, and in February and March, there is a definite rise in oxygen consumption, evidently correlated with the quickened growth and activity characteristic of May flies at this season.

Does other behavior of the nymphs in any way express their "low" winter condition? When its light response changes from positive to negative, the metabolism of the May-fly nymph *Epeorus* is lowered (Allee and Stein, 1918). In a series of preliminary tests made upon the light responses of the *Hexagenia recurvata* nymphs under various conditions, there was some evidence that, on the average, they were more negative to light in October and February than in March and May. A possible correlation is thus indicated between their phototropism and respiratory metabolism. A relationship of this kind has been suggested by Allee and Stein (1918) in nymphs of the May flies *Epeorus* and *Leptophlebia*. Using potassium cyanide as an index of metabolism, they demonstrated a correlation between phototactic reaction and metabolic condition. In their normal behavior Holmquist (1928) found that, in the cold, muscid flies were inactive and had a negative reaction to light; whereas in the warmth, they were more active and were positive to light. So far as they have been studied here, the responses of *Hexagenia recurvata* nymphs also indicate that they manifest their lower metabolism by more negative reactions to light during the winter and their higher metabolism by more positive ones in the spring.

Absence of molting may be taken as a manifestation of low metabolism in nymphs of *Hexagenia recurvata*, as it has been shown to be in the dragon fly, *Anax junius*, by Calvert (1929) and as discussed for insects by Singh-Pruthi (1925). No nymphs molted in the laboratory cages from December 3 to April 29. Neither were there any recently molted nymphs among those collected from Lithia Springs Brook between November 15 and March 15. On the other hand, of those taken on May 3, 17 per cent were recently molted ones. The oxygen consumption of the nymphs showed that their metabolism started to rise in the latter part of January. Its continued rise is indicated by the nymphs' greater positiveness to light in March and the frequent appearance of molted skins after April 29.

All nymphs of *Hexagenia recurvata* showed greater resistance to laboratory conditions between November and February, their period of lowered oxygen consumption, than they did before and after those months. In the examinations of the cages from November 12 to January 8, 4.96 was the highest percentage of nymphs found dead. But beginning on January 21 the mortality rose from 11.45 per cent to 38.15 per cent on April 15.

Experiments and natural behavior have shown that sluggish animals can endure an environment which would be fatal to them if their metabolism were more rapid. For example, animals with a higher metabolism are more susceptible to a strong solution of potassium cyanide than those with a lower one (Allee and Stein, 1918; Child, 1919; Hyman, 1919). It seems therefore that in *Hexagenia recurvata* the earlier resistance, and later lack of resistance, to unfavorable conditions of the laboratory is also a manifestation of their lowered metabolism during the winter and its rise toward spring.

It has already been shown that there is a metabolic rhythm in these nymphs which is, to a considerable degree, independent of temperature. Annual metabolic cycles are more evident in other insects undergoing a diapause, a period of low metabolism resulting in complete quiescence occurring independently of temperature. Nymphs of *Hexagenia recurvata* have no diapause, nor can they be said to be intermittently inactive. Yet, their period of low oxygen consumption, even though occurring during their winter of more or less continuous activity, is suggestive of the more striking hibernation phases of other insects.

SUMMARY

1. Nineteen hundred and eighty-five nymphs of *Hexagenia recurvata* were collected from a spring-fed brook in eighteen collections, made mainly in the winter and early spring (September 19 to May 3). Observations were made upon their behavior in their habitat and under laboratory conditions in which they were kept from 10 days to 2 months.

2. Experimental tests of the oxygen consumption of a total of 205 laboratory-kept and recently collected nymphs were conducted from

October 27 through March 17. Three tests made in October and November were counted as preliminary; and the results of nine, performed in December, January, February, and March, are reported here.

3. The oxygen consumption of nymphs kept in the laboratory from 10 days to 2 months was less than that of nymphs collected from their own habitat and tested within 24 hours.

4. Between December 1 and January 13 the oxygen consumption of all nymphs was lowered; between January 29 and March 17 it rose. The nymphs thus exhibit a late-winter rise in oxygen consumption.

5. In all laboratory-kept nymphs and in recently collected ones tested at temperatures higher than that of their own habitat, a "pickup" in oxygen consumption occurred in the last part of January. In recently collected nymphs tested at the temperature of their own habitat, it occurred in February.

6. In experimentally varied temperatures the oxygen consumption was directly proportionate to the temperature, a Q_{10} of from 2.6 to 3.3 being obtained.

7. In their rate of oxygen consumption nymphs of *Hexagenia recurvata* are independent of the oxygen tension from 9.75 to 2.34 cc. of oxygen per liter of water, despite accumulating carbon dioxide. They may survive when there is but 0.50 cc. of oxygen per liter of water.

8. Among other evidences of low metabolism was an absence of molting from December 3 to the end of April. Molting was frequent, however, during May and October.

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THE EFFECTS OF CARBON DIOXIDE, THE HYDROGEN ION, CALCIUM, AND EXPERIMENTAL CONDITIONING ON RECONSTITUTION IN EUPLANARIA DOROTOCEPHALA.[1]

(Six figures)

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THE effects of acids on physiological processes have long been of interest to investigators. One has but to look at the tremendous amount of literature that has accumulated on the subject to realize that the H-ion is of prime importance. It is no doubt true that many biological phenomena have been attributed directly to the external H-ion when in reality the relationship may be very indirect. Alterations of the H-ion concentration may cause an increase or a decrease in the solubility of other substances in the solution, and this factor alone may be responsible for many phenomena that have been stated as being results of the direct action of the H-ion.

The effects of external CO_2 have often been confused with the effects of the external H-ion. The alteration of physiological processes of organisms in natural waters to which acid has been added has often been stated as being the result of H-ion rather than of the CO_2 which may have been liberated on acidifying the water.

In general it has been found that increased H-ion and CO_2 concentration in the external medium serve as inhibitors to biological processes. In recent years much work has been done in altering reconstitutional and embryological development through the use of various inhibiting agents applied externally. It has been found, in general, that agents which tend to inhibit development in organisms do so differentially. In other words, the more active regions may be inhibited to a greater extent than the less active regions. Certain phenomena of twinning, exogastrulation, and various other teratological processes are evidently examples of differential inhibition.

[1] The author wishes to thank Professor C. M. Child for much valuable advice and criticism received throughout the progress of this work.

In this investigation we are concerned chiefly with the effects of the H-ion and CO_2 on reconstitutional development.

THE PROBLEM

Child (1911*a*) pointed out that transverse pieces of *Euplanaria dorotocephala*, of a limited size and from the more posterior levels of the first zooid, reconstituted in a large number of cases into headless individuals or individuals which showed various degrees of head inhibition. The frequency of inhibited head-forms and the degree of inhibition were found to be related to the level from which the piece was taken and to its length. Short pieces and pieces from the posterior levels of the anterior zooid showed the greatest inhibition in head-form. Child has placed the reconstituted head-forms in the following classes, corresponding to the degree of inhibition:

Normal.—Head triangular with two symmetrically placed eyespots and with auricles at lateral margins.

Teratophthalmic.—Head normal in shape and with auricles in normal position, but with eyespots showing all degrees of approximation to the median plane from two distinct eyespots with pigment connected to complete cyclopia.

Teratomorphic.—Head more or less rounded in outline with single or apparently single median eye; auricles more or less anterior and showing all degrees of approximation to the median plane up to a single median auricle.

Anophthalmic.—Heads rudimentary without eyespots and with or without a single median auricle.

Acephalic.—Head completely absent.[2]

It subsequently has been found and sustained by Child and others that the inhibition of normal head formation in transverse pieces of *Euplanaria* is related to some inhibitory influence which arises from the posterior cut surface of the isolated piece. This factor inhibiting head development is most effective during the first 24 hours after section; yet, it may still be evidenced, as shown by delay of anterior

[2] Since Child (1911*a*, 1911*b*, 1911*c*, 1912, 1914*a*, 1914*b*, 1916, 1920, 1924), Child and Watanabe (1935), and others (Behre, 1918; Buchanan, 1922; Hinrichs, 1924) have repeatedly described and figured the different head types in *Euplanaria*, it does not seem necessary to present either drawings or photographs of them here.

section, after 3 or 4 days (Child and Watanabe, 1935). It is quite evident that this inhibiting action of the posterior cut surface is not entirely the result of stimulation by section, since this stimulation, as indicated by increased respiration and susceptibility, disappears within a few hours (Child, 1914*a*; Robbins and Child, 1920; Hyman, 1923). It is probable that the cellular activity associated with the early stages of tail development also plays a part in the inhibition. The inhibiting factor is apparently largely or wholly nervous in character and can be decreased or obliterated by delay of posterior or anterior section or by section under anesthesia (Child, 1914*b*; Buchanan, 1922; Child and Watanabe, 1935; Watanabe, 1935*b*).

In dealing with the various types of heads that arise from lots of reconstituted worms, the term "head frequency" has been used. By head frequency is meant the frequency of the various forms that reconstitute from lots consisting of pieces of a certain length and from a certain body-level of animals of the same length. If a certain lot of pieces gives a preponderance of normal and near-normal animals, we speak of the lot as having a high head frequency. If a lot gives a majority of headless or strongly inhibited head-forms, we speak of it as having a low head frequency.

Many workers have found that pieces of planarians, which would normally give a low head frequency, gave an increased head frequency when allowed to reconstitute in certain slightly toxic solutions. Reagents which have been used to increase head frequency in the posterior levels of the first zooid are dilute KCN (Child, 1916), chloretone, chloroform, chloral hydrate, ether, ethyl alcohol (Buchanan, 1922), and caffeine (Hinrichs, 1924). Mechanical stimulation (Child, 1920) and abrupt change in temperature from 20° to 29° C. (Behre, 1918) have also been found to increase head frequency.

Child (1911*a*, 1912, 1916) has shown that the head frequency of pieces from anterior levels and of long pieces could be decreased by lowering the temperature or by allowing the pieces to reconstitute in dilute solutions of metabolic end-products, ethyl alcohol or KCN, for varying intervals of time. Behre (1918) found that animals, taken from a living temperature of 20° C., sectioned, and allowed to reconstitute in a temperature of 10° C., gave a decrease in head frequency. Buchanan (1922) found a slight decrease in the head frequency in

anterior pieces with short time exposure to anesthetics. Hinrichs (1924) found that anterior pieces showed a decreased head frequency with initial exposure of 24 hours to M/200 caffeine.

In general it would seem that those agents which increase head frequency do so either by inhibiting the effect from the posterior cut surface or by stimulating the head-forming cells at the anterior cut surface. A decrease in head frequency appears to be the result of an increased effect of the inhibiting factor or a decrease in the activity of the head-forming cells at the anterior cut surface.

Since the H-ion and CO_2 have been regarded as important factors in many biological processes, it was suggested that the reconstitution of *Euplanaria dorotocephala* in different concentrations of H-ion and CO_2 might well be investigated. Experiments were also suggested which dealt with the possible effects of calcium in antagonizing the H-ion and with the experimental alteration of physiological condition of animals prior to section.

MATERIAL AND METHODS

The material used in this investigation was the common flatworm, *Euplanaria dorotocephala*, which is found in great numbers in springs of the Valparaiso moraine to the south and west of Chicago. The chief localities for the collection of worms during this work were Cary, Illinois; Rockford, Illinois; and Valparaiso, Indiana. The worms were kept in large granite pans in the laboratory for several weeks before the experiments were performed. They were fed twice a week on beef liver, and the water was changed on alternate days.

Except when it was desired to bring out the effects of recent feeding, the worms were starved for approximately 2 weeks before they were used in the experiments. (Animals which have been recently fed usually show greater variability in head frequency, as well as a greater susceptibility.) Although 12–14, 14–16, and 15–17 mm. animals were used, by far the most experiments were performed with animals 14–16 mm. in length. Unless particular attention is called to this fact, it may be assumed that 14–16 mm. animals are being used.

Lots of 65 animals of the same length and breadth were chosen from the same stock for tests and controls. The test animals were al-

ways washed twice in the test solution before sectioning. Test animals were sectioned in their respective test solutions, while control animals were sectioned in well water.

In the head-frequency experiments the animals were cut in transverse sections in the manner shown in Figure 1. All the pieces were approximately one-eighth the length of the animal, exclusive of the head. Only the anterior zooids were used, and after a little practice it was possible to cut the pieces with a great deal of exactness. Of the 65 pieces from each region, 15 showing the greatest variations in length were discarded. This procedure considerably decreased any error related to faulty cutting.

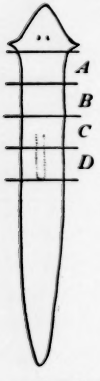

FIG. 1.—Levels *A*, *B*, *C*, and *D* used in all head-frequency experiments. Each section is approximately 1/8th the length of the animal, exclusive of the head. Head and region posterior to the mouth were discarded.

After section the pieces from the different levels were placed in 1-liter Erlenmeyer flasks which were filled with the test and control solutions. The flasks were tightly stoppered and left in the dark at room temperature until the pieces reconstituted. The test and control solutions were changed daily in a great many of the experiments, but in the majority of cases it seemed advisable not to stimulate the pieces by changing the solution. Other reasons for not changing the solutions in which the worms are reconstituting will become clear as the individual experiments are described.

After 2 weeks the reconstituted animals were washed from the flasks and examined under a binocular microscope. They were given arbitrary values according to the type of head as follows: normal, 100; teratophthalmic, 80; teratomorphic, 60; anophthalmic, 40; acephalic, 20. These arbitrary values were multiplied by the frequency of the different head-forms, and the sum of all was divided by the total number of *living* animals to obtain the "head-frequency index." (This "head-frequency index" is identical with the "mean" described by Child and Watanabe, 1935.)

The test solutions were made up in 16-liter quantities as follows:

1. *Carbon dioxide solutions.*—Since the well water in the laboratory contains a large quantity of bicarbonates, it was decided to make use of the CO_2 that was liberated when HCl was added to the water. It was found that enough acid added to the well water to change the pH from 7.5 to 5.4 released approximately 60 cc. of CO_2 per liter. Changes in pH from 7.5 to 6.0, 7.5 to 6.6, and 7.5 to 7.0 released 45 cc., 18 cc., and 8 cc. of CO_2 per liter, respectively. The CO_2 liberated is, of course, not all in the gaseous form but is largely in such forms as H_2CO_3, $^{-}HCO_3$, and $^{=}CO_3$. If the solution was allowed to stand in a container open to the air for a few hours, the CO_2 was expelled and the pH of the solution rose to 7.0 or above. As this method for obtaining CO_2 in solution was used, all tests involving the use of this reagent are recorded in terms of their pH values.

2. *Hydrogen ion solutions.*—It was highly desirable that the well water in the laboratory be used in making up the test solutions, as it contains the proper salts and was used for the controls. In order to be rid of the effect of CO_2 when testing the effect of pH, it was necessary to first free the water of bicarbonates. Smith and Clowes (1924) found that the bicarbonate could adequately be removed from sea water by adding enough concentrated HCl to break down all carbonates and bicarbonates and then removing the CO_2 by pumping air through the solution. Hyman (1925) used this method for removing the bicarbonates from well water.

The method used in these experiments for the removal of bicarbonates was similar to Hyman's method. Four and one-half cubic centimeters of concentrated HCl were added to 16 liters of well water, and air was bubbled through this solution for 24 hours. At the end of this time the CO_2 in the water was in equilibrium with the air and was found to be of no importance. The buffering capacity of this carbonate-free water almost entirely disappeared. The pH was adjusted to the desired values by adding minute quantities of M/10 HCl or M/10 NaOH. The exact pH value was determined by a quinhydrone apparatus supplemented with a series of La Motte color indicators.

THE EFFECT OF CARBON DIOXIDE ON HEAD FREQUENCY AND VIABILITY

A number of experiments were performed in determining the exact concentrations of CO_2 the animals could tolerate. This procedure was necessary since a large number of experiments have shown that those concentrations which are most effective in altering head frequency are usually very near the lethal level. In fact, it has been found that solutions which cause cytolysis of a small percentage of the experimental pieces are usually the most effective.

Experiments in which intact animals 14–16 mm. in length were placed in CO_2 solutions ranging from 4.8 to 7.0 showed that cytolysis in the head regions often took place in concentrations below pH 5.2. Some worms even underwent complete disintegration at these concentrations. One-eighth pieces were quite susceptible to pH 5.2 and usually cytolyzed within a few hours. A concentration in which only a few pieces died was found at pH 5.4–5.6.

It was noted with much interest that animals which had lost only their heads in concentrations of pH 5.2 or below would reconstitute new normal heads in exactly the same solutions that had caused the cytolysis of the old head. It was also noted that those animals which had lost their heads, as well as animals whose heads had remained intact, often underwent fission in high concentrations of CO_2. The percentage of fission was apparently closely related to the size and general physiological condition of the worms.

Another phenomenon, which was noted at this time and later made use of in experiments on "conditioning," was the acquiring of tolerance by intact animals to CO_2. It was found that animals which had been kept in solutions of pH 5.4–5.6 for a few days could live, with no apparent injury, when transferred to solutions of pH 5.2. If kept in the latter solution for a few days, they could then be transferred to pH 5.0 without cytolysis resulting. In other words, animals living in certain concentrations of CO_2 for a time will acquire a tolerance for this reagent and, in doing so, will be able to survive solutions that are toxic to animals taken directly from well water, pH 7.5.

In the experiments on head frequency described in this section only worms which had been kept in freshly drawn well water until the time for experimentation were used. In the early experiments the solutions were changed daily; but, because of the large quantity of solution, as compared to the pieces, and the fact that there was little or no change in the solutions during the reconstitutional period, this was given up as time-consuming. Other reasons for not changing the solutions were that the type of head appears to be determined by at least 2 or 3 days after section and the stimulation of pieces in

TABLE I

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CARBON DIOXIDE SOLUTIONS

(Head types and deaths given in percentages)

	Well Water (Control)				Carbon Dioxide Solution, pH 5.4–5.6			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Normal	92.0	34.6	7.3	2.6	72.0	60.0	18.6	13.3
Teratophthalmic	8.0	40.0	5.3	0.6	5.3	14.0	20.0	8.0
Teratomorphic	.....	2.0	4.6	.....	1.3	0.6	2.6	4.6
Anophthalmic	.....	10.0	21.3	10.3	.....	4.6	15.3	6.0
Acephalic	.....	10.6	60.6	86.6	1.3	10.0	29.3	22.6
Dead	.....	2.0	0.6	.....	20.0	10.0	14.6	45.3
Index	98.4	75.9	35.3	23.9	96.6	84.6	55.8	53.6

changing the solutions might present additional, not completely controllable factors.

In Table I are presented the head frequencies of three different experiments involving 150 test and 150 control worms. These experiments were performed on three different stocks and at three different times; but as each experiment shows the same relationship between tests and controls, these three different experiments are grouped together. It can readily be seen from this table that the head frequencies are distinctly higher in *B*, *C*, and *D* pieces which have reconstituted in the test solution. The relationship between tests and controls is shown graphically in Figure 2, in which the head frequency indices are plotted against body-level.

Another fact brought out in Table I is the different percentages of viability of pieces from different levels. The data at least indicate that *A* and *D* pieces are more susceptible to the toxic effect of CO_2 than are pieces *B* and *C*. It may be that *A* pieces are more susceptible because of the normal higher activity of that particular level. The high susceptibility of *D* pieces may be attributed to the tremendous increase in respiratory metabolism and in susceptibility which these levels undergo following section. The presence of the pharynx in *D* pieces, may also have something to do with the greater susceptibility.

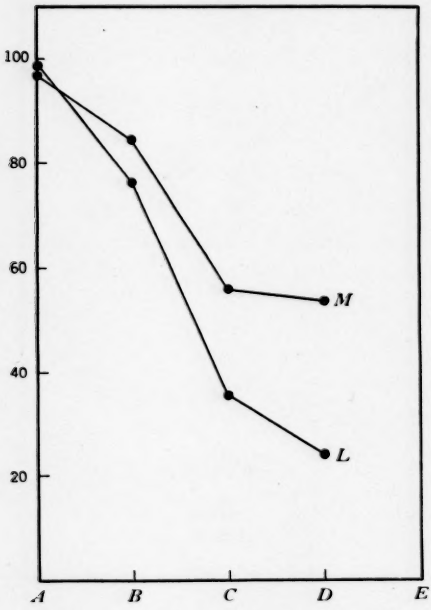

FIG. 2.—Head frequencies in CO_2 solution (*M*) and well water (*L*). Graph is plotted with the head-frequency indices as ordinates and body-levels as abscissae.

Experiments on head frequency were performed in which the concentrations of CO_2 were pH 5.4, 6.2, and 6.6. The head-frequency indices and the percentage of viability are shown in Table II. These worms were of the same size and from the same stock. They were sectioned at the same time and serve as adequate controls for each other.

It will be noted that pieces which have reconstituted in pH6.2 show a head frequency as high as, or possibly higher than, those which have reconstituted in pH 5.4; yet the number of deaths is practically zero. Solutions with a pH of 5.4 contain approximately 60 cc. of CO_2 per liter, while those with a pH of 6.2 contain approxi-

mately 38 cc. per liter. It appears from these data that pH values below 6.2 are more effective in increasing the number of deaths than the head frequency. At pH 6.6 the effect of CO_2 is still evident at *C* and *D* levels.

That this effect we have been describing is actually due to CO_2 rather than the H-ion is shown in Table III, in which the head-fre-

TABLE II

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN VARIOUS CONCENTRATIONS OF CARBON DIOXIDE

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water (control)	97.4	82.4	39.2	27.2	100.0	98.0	100.0	100.0
CO_2 solution, pH 5.4	91.5	89.2	64.4	58.7	80.0	82.0	72.0	62.0
CO_2 solution, pH 6.2	99.6	96.4	77.6	51.0	100.0	100.0	100.0	98.0
CO_2 solution, pH 6.6	97.4	81.2	47.0	42.0	100.0	100.0	100.0	100.0

TABLE III

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CERTAIN NON-EFFECTIVE HYDROGEN ION SOLUTIONS

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water (control)	98.8	81.6	41.0	31.8	100.0	100.0	72.0	100.0
Carbonate-free well water, pH 7.5	98.8	68.0	36.0	30.8	100.0	100.0	100.0	100.0
Carbonate-free well water, pH 5.6	99.0	78.0	34.6	27.4	100.0	100.0	100.0	100.0

quency indices of worms which have reconstituted in carbonate-free well water, pH 5.6 and 7.5, and in unaltered well water (control solutions), pH 7.5, are compared. According to Table III, the lots in carbonate-free well water at pH 7.5 and 5.6 show slightly lower head frequencies than the control, but the significance of the differences is doubtful. That the effect of H-ion alone at these concentrations is unimportant is fully confirmed by later experiments.

THE EFFECT OF THE HYDROGEN ION ON HEAD FREQUENCY AND VIABILITY

All test animals dealt with in this section were allowed to reconstitute in carbonate-free well water which was prepared by the method described earlier in this paper.

Experiments on intact animals submitted to different concentrations of the H-ion show that this reagent has little effect between pH values of 7.2 and 4.2. Below 4.1 all of the animals usually cytolyzed. There was no evidence at this time of any acclimation to high concentrations, and no increase in fission rate was noted. If animals are left in pH 4.15 for several days, the pigmentation darkens and the tissues appear somewhat swollen. In alkaline solutions the animals died at a pH of approximately 10.0. In these solutions there was no evidence of any physiological changes before death.

A series of fifteen experiments was devised in which the head frequencies of the different levels of 50 animals were determined at every 0.4 pH value between pH 4.2 and 9.6. The solutions were changed daily throughout the entire reconstitutional period. The worms used in these experiments were from the same lot and were sectioned at approximately the same time. From these fifteen experiments the only concentration that gave any evidence of inducing an increase in head frequency was pH 4.2. There was no effect whatever between pH 4.6 and 7.0. From 7.0 to 9.6 there appeared to be a slight decrease in the head frequencies of reconstituted *C* pieces.

As a pH of approximately 4.2 seemed to be quite important in the alteration of physiological conditions in reconstituting pieces, twelve different experiments, involving over 1,200 worms, were performed at pH ranges from 4.15 to 4.30. Each experiment consisted of 50 test and 50 control worms. These animals were from different localities and were collected at different times throughout the year. Although the head frequencies of the animals from different stocks did not always coincide, the head frequencies of the tests were always well above the head frequencies of the controls. The data from these different experiments were therefore grouped together in Table IV.

It will be noted from Table IV that the percentage of normal worms is much greater in the tests than in the controls, except in *A* pieces, and that the percentage of acephalic forms in the tests is

much smaller. A comparison of the head-frequency indices of worms which have reconstituted in critical concentrations of CO_2 (Table I) and of H-ion (Table IV) indicates that in these concentrations the H-ion may be more effective in increasing head frequency than CO_2. It will also be noted that the death frequency in the tests at the different levels corresponds to the death frequency of pieces in high concentrations of CO_2.

TABLE IV

HEAD FREQUENCY AND VIABILITY OF *Euplanaria dorotocephala* IN CARBONATE-FREE WELL WATER

(Head types and deaths given in percentages)

	Well Water (Control)				Carbonate-Free Well Water, pH 4.15–4.3			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Normal	91.1	43.7	13.0	8.3	76.6	51.2	35.8	35.7
Teratophthalmic	7.7	27.7	11.3	7.5	6.2	22.7	19.2	14.3
Teratomorphic	1.0	2.3	4.2	3.5	0.2	2.7	3.8	6.3
Anophthalmic	0.2	9.6	20.2	9.0	0.2	4.1	13.8	9.3
Acephalic	.....	16.6	50.8	71.2	.....	7.5	16.8	13.2
Dead	.....	.....	0.5	0.5	16.6	11.8	10.5	21.2
Index	97.9	74.4	43.0	34.3	98.1	84.0	69.6	72.7

Pieces that have reconstituted under conditions of high H-ion concentrations (pH 4.15) show morphological characteristics which are quite different from the controls. The pigmentation in the old tissues of the reconstituted test worms usually becomes very dark and diffuse. The new outgrowth may remain almost entirely unpigmented for a considerable length of time. Stunting of new tissue in both head and tail regions has been noted in such high concentrations of the H-ion.

CALCIUM ANTAGONISM TO THE HYDROGEN ION

Much work has been done in the past on the antagonism of ions. It has been found, in general, that the injurious effect of one ion may be obliterated by another ion even though the other ion alone may produce injuries. Divalent ions have been found to antagonize the actions of monovalent ions to a great extent. Calcium, particularly,

has been used in many fields of biological investigation to antagonize the injurious effects of sodium and potassium.

It has been established, in this work, that the H-ion in high concentrations has the property of altering reconstitutional development in *Euplanaria*. The question arises as to whether or not the effect of this ion can be antagonized by increasing the calcium content of the test solutions.

Preliminary experiments were carried out on intact worms in which from 0.1 to 1.0 gm. of calcium ($CaCl_2$, anhydrous) were added per liter of test solution. The pH of the solutions was approximately 4.0. It was found that animals in solutions of the same pH would live a much longer time if the calcium concentration was increased. It apparently made little difference whether the calcium concentration was increased 0.1 or 1.0 gm. per liter.

A series of experiments was next conducted to determine if calcium could antagonize the effect of H-ion on head frequency. Six experiments were performed, which involved 900 worms from the same stock. Each experiment consisted of a control in well water, pH 7.5, a control in carbonate-free well water, pH 4.15, and a test in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The results of these six experiments are tabulated in Table V. It will be noted from this table that worms allowed to reconstitute in carbonate-free well water at pH 4.15 have a characteristically higher head frequency than those which have reconstituted in well water, pH 7.5. This is not true in the case of pH 4.15, where the calcium concentration of the solution has been increased. Instead, the head frequency in these solutions approaches very closely the head frequency obtained in the well-water controls. In other words, the addition of calcium to these concentrations of the H-ion almost completely antagonized its effect on head frequency. Not only is this effect of the H-ion on head frequency decreased with calcium, but the death percentages of the pieces are likewise decreased under the same conditions.

In the light of the foregoing observations, a head-frequency experiment was performed, using as control solutions well water, pH 7.5, and carbonate-free well water, pH 4.1. The test solution was carbonate-free well water, pH 4.1, plus 0.5 gm. of $CaCl_2$ per liter.

The head frequencies and viability in this experiment are recorded in Table VI. It will be seen, in this case, that calcium antagonizes to a high degree the death-rate in high concentrations of H-ion but that the increase in head frequency is only partially antagonized.

TABLE V

ANTAGONIZING ACTION OF CALCIUM ON EFFECTIVE CONCENTRATIONS OF THE HYDROGEN ION

(Head types and deaths given in percentages)

	Well Water (Control)				Carbonate-Free Well Water, pH 4.15				Carbonate-Free Well Water, pH 4.15, Plus 0.5 Gm. $CaCl_2(L)$			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Normal	92	21	3	4	78	40	21	23	93	36	4	4
Teratophthalmic	7	28	3	1	4	28	16	8	5	30	4	2
Teratomorphic	1	3	1	1	....	3	6	6	....	5	4	1
Anophthalmic	....	16	12	4	....	7	16	12	....	11	23	7
Acephalic	....	31	80	88	....	14	32	20	....	20	65	83
Dead	....	1	....	2	18	9	10	31	2	....	....	2
Index	98	59	27	25	99	76	55	61	99	70	32	27

TABLE VI

THE PREVENTION OF DEATH BY THE HYDROGEN ION WITH CALCIUM

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water (control)	97.9	44.5	21.6	20.8	98.0	98.0	100.0	100.0
Carbonate-free well water, pH 4.1	.....	.....	90.0	.....	0.0	0.0	8.0	0.0
Carbonate-free well water, pH 4.1, plus 0.5 gm. $CaCl_2$ (L)	99.0	81.7	40.0	42.9	74.0	94.0	92.0	96.0

It was of interest in considering the subject of ionic antagonism to test the viability and head frequency of highly susceptible animals (those fed 48–72 hours prior to section) in concentrations of H-ion, pH 4.15, with and without additional calcium. The results are shown in Table VII. In this case the death-rate is considerably antagonized,

while the head frequency is only partially antagonized by the action of calcium.

In order to test the effect of increased concentrations of calcium, aside from the antagonistic action on the H-ion, a series of experi-

TABLE VII

THE EFFECTS OF CALCIUM AND THE HYDROGEN ION ON HIGHLY SUSCEPTIBLE ANIMALS

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water (control)	100.0	65.3	34.1	28.3	90.0	84.0	88.0	96.0
Carbonate-free well water, pH 4.15	100.0	80.0	56.0	46.6	8.0	4.0	10.0	12.0
Carbonate-free well water, pH 4.15, plus 0.5 gm. $CaCl_2$ (*L*)	99.2	64.0	45.3	41.3	100.0	90.0	98.0	90.0

TABLE VIII

THE ACTION OF CALCIUM ALONE ON HEAD FREQUENCY AND VIABILITY IN *Euplanaria dorotocephala*

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water, pH 7.5 (control)	99.2	64.4	22.5	20.0	100.0	100.0	96.0	96.0
Well water, pH 7.5, plus 0.5 gm. $CaCl_2$ (*L*)	100.0	71.2	24.7	21.2	100.0	100.0	100.0	100.0
Carbonate-free well water, pH 4.15	100.0	87.6	64.5	51.2	98.0	100.0	98.0	68.0
Carbonate-free well water, pH 4.15, plus 0.5 gm. $CaCl_2$ (*L*)	99.6	77.2	32.2	26.4	100.0	100.0	98.0	100.0

ments was performed in which the test solution was well water, pH 7.5, plus 0.5 gm. of $CaCl_2$ per liter. The control solutions were well water, pH 7.5, carbonate-free well water, pH 4.15, and carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The results, which are tabulated in Table VIII, indicate that calcium alone may slightly increase the head frequency. In this case it apparently has little effect on viability.

One head-frequency experiment was performed in which $CaCl_2$ (0.5 gm. per liter) was added to a test solution of CO_2 pH 5.4. This experiment was adequately controlled with solutions of CO_2, pH 5.4, and well water, pH 7.5. It was found that calcium did not antagonize the effects of CO_2 either by decreasing the head frequency or the death percentages.

THE PHYSIOLOGICAL CONDITION OF *Euplanaria* IN NATURE

Throughout this investigation it has been found that control worms of the same size but from different stocks may yield very different head frequencies.

It has also been noted that worms from the same stock vary considerably from time to time as regards head frequency. Animals collected in the summer may show a different head frequency than those collected in the winter. It has been observed that worms in "poor" condition (i.e., many cytolyzing in the stock pans) usually show a higher head frequency than worms from the same stock under better conditions.

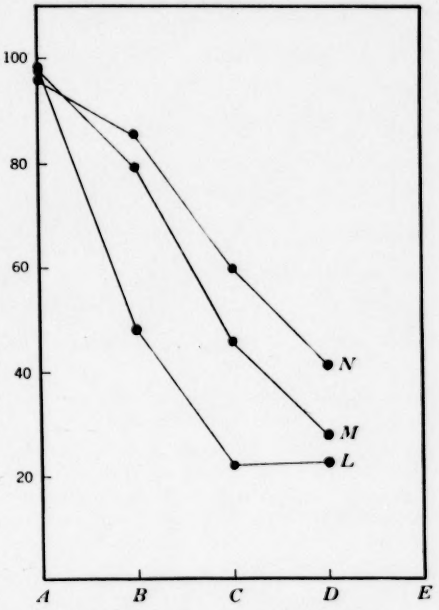

FIG. 3.—Head frequencies of different stocks of *Euplanaria dorotocephala* which have reconstituted in well water. Stocks were from Valparaiso, Indiana (*L*); Cary, Illinois (*M*); and Rockford, Illinois (*N*.)

Worms of the same size and kept under the same laboratory conditions may behave differently if they are originally from different localities. In Figure 3 are plotted the head-frequency indices of 150 control worms from each of three widely separated places.

It is not intended to imply by this graph that worms from different localities are persistently different. The head frequencies of worms (from any one locality) at different times in the year may vary as much as the three different head frequencies shown in the graph. The only object of the graph is to show that the different lots of worms used in our experiments may be in different physiological conditions and that these conditions are expressed through the head frequencies. This difference in physiological condition must have been impressed upon the worms by their different environments prior to section.

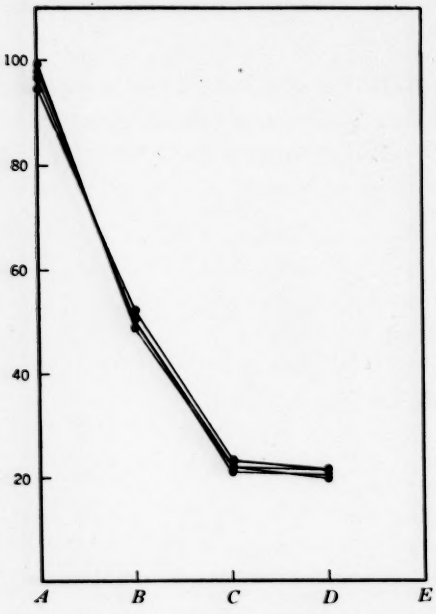

FIG. 4.—Head frequencies of four lots of worms from the same stock. They were identical in size and condition and were sectioned at approximately the same time. All were allowed to reconstitute in well water at the same temperature. (Worms were from Valparaiso, Indiana.)

But is there any pronounced difference in head frequency of different lots of worms of the same size, from the same stock, collected at the same time, sectioned at approximately the same time and kept under the same laboratory conditions? The answer to this question is given in Figure 4, in which the head-frequency indices of four control lots of 50 worms per lot, which met all the requirements, are plotted. It must be concluded from these data that like worms give like head frequencies and that all experiments on the alteration of head frequency have been adequately controlled.

It has been repeatedly observed that worms with controls of relatively high head frequency respond to the effects of such reagents as

H-ion and CO_2 with a greater increase in head frequency than stocks in which the controls show a low head frequency. Apparently animals in good condition (with low head frequency), although more susceptible than those in poor condition to directly lethal agents, are more capable of maintaining approximately normal internal conditions or of regulating these conditions when subjected to CO_2 or H-ion in the non-lethal concentrations used in these experiments. In other words, the animals in good condition tolerate these concentrations better, and so appear to possess a greater physiological stability within the experimental limits than those in poor condition.

THE EXPERIMENTAL ALTERATION OF PHYSIOLOGICAL CONDITION IN *Euplanaria*

Since worms of the same size but from different environments yield different head frequencies under the same experimental conditions, a number of experiments were performed to determine whether the physiological conditions of animals could be altered in the laboratory in a short time by experimental means.

Three different methods for altering the physiological condition of the animals were tested. These were as follows:

1. Lots of 500–700 worms (14–16 mm.) were placed in a tightly stoppered 18-liter bottle in a solution of CO_2, pH 5.6–5.7, for 4 days. This was followed by 6 days in CO_2 solution, pH 5.05–5.2. At the end of the conditioning period the worms were used for experimentation.

2. Lots of 500–700 worms (14–16 mm.) from the same stock as the worms in (1) were placed in carbonate-free well water, pH 4.15, in a tightly stoppered 18-liter bottle. The worms were left in this solution 9–10 days before sectioning for head frequency. The solution was changed daily.

3. Lots of worms of the same size and from the same stock as (1) and (2) were placed in well water, pH 7.5, in a tightly stoppered 18-liter bottle. The worms were kept under these conditions for 10 days prior to sectioning. The water was not changed from the beginning to the end of the conditioning period.

A control lot of 500–700 worms of the same size and from the same stock was maintained in an open pan, and the water changed daily.

Animals treated in the foregoing manner were taken directly from the experimental solutions, sectioned, and allowed to reconstitute in well water, pH 7.5, in carbonate-free well water, pH 4.15, and in carbon-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. The head frequencies were compared with non-treated control worms which had been submitted to the same test solutions during reconstitution.

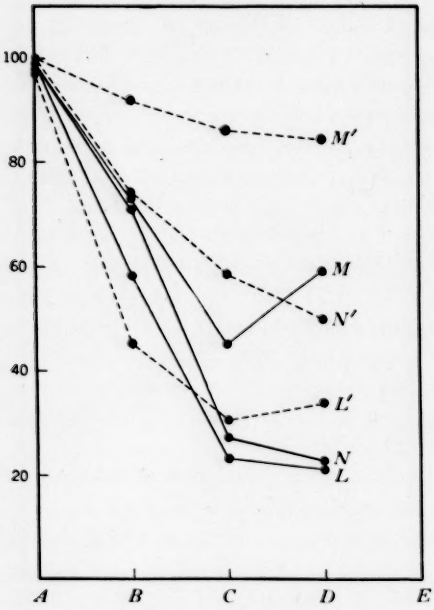

FIG. 5.—Head frequencies of CO_2 conditioned worms (broken lines) under different experimental and control conditions, as compared to non-conditioned worms. *L*, non-conditioned worms which have reconstituted in well water. *L′*, conditioned worms which have reconstituted in well water. *M*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *M′*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15. *N*, non-conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter. *N′*, conditioned worms which have reconstituted in carbonate-free well water, pH 4.15, plus 0.5 gm. of $CaCl_2$ per liter.

1. *The effect of carbon dioxide on physiological condition.*—Animals conditioned with CO_2 gave by far the best results and showed clearly how the physiological gradients may be altered in intact animals before section.

Euplanaria conditioned to CO_2 and allowed to reconstitute in well water shows a higher head frequency in *C* and *D* pieces. In this experiment the conditioned *B* pieces may show a lower head frequency than the controls, as shown in Figure 5 (*L*, *L′*).

Carbon dioxide conditioned worms which had reconstituted in carbonate-free well water, pH 4.15, showed a considerably higher

head frequency than non-conditioned worms in the same solution (Fig. 5 *M*, *M'*). In the non-conditioned worms the percentages of deaths were greater in *A* and *D* pieces. In the conditioned worms the death percentages (although as high) were not distributed in a regular fashion.

The non-conditioned worms reconstituting in pH 4.15 plus 0.5 gm. of $CaCl_2$ per liter (Fig. 5 *N*) give a head frequency almost as low as the non-conditioned worms reconstituting in well water (Fig. 5 *L*). In the conditioned worms (Fig. 5 *N'*), however, the antagonism of calcium to the H-ion decreases the head frequency below that of conditioned worms in H-ion alone (Fig. 5 *M'*), but it remains far above that of the non-conditioned worms (Fig. 5 *N*). Evidently the antagonism of calcium to the H-ion is not complete as regards head frequency in animals conditioned to CO_2.

An interesting series of head-frequency experiments was performed on a separate lot of CO_2 conditioned worms in which pieces from the different levels were placed in CO_2 solution, pH 5.35, for given intervals of time after section. The pieces were then returned to well water, pH 7.5. In this series the reconstitution of the conditioned worms took place in (1) well water, pH 7.5, for the entire period, (2) CO_2 solution for the first 24 hours, (3) CO_2 solution for the first 48 hours, (4) CO_2 solution for the first 72 hours, and (5) CO_2 solution for the entire period. The head-frequency indices are presented in Table IX.

It will be noted from these data that all levels which have undergone CO_2 treatment, either before or after section, show an increased head frequency. It is of particular interest that conditioned pieces show a much higher head frequency when submitted to CO_2 for only 24–48 hours after section than with longer periods. (This effect may be because of a differential recovery or an activation of the head-forming cells that has been brought about by the change from an inhibiting solution to well water. If this is the case, we may assume that the exposure time was sufficient to inhibit the posterior-cut-surface factor.)

Animals conditioned in CO_2, sectioned, and allowed to reconstitute for the entire period in CO_2 solution show a lower head frequency than would be expected from the results with non-condi-

tioned worms. This fact suggests that an acquired tolerance to CO_2 before section may result in less effect of CO_2 after section.

2. *The effect of the hydrogen ion on physiological condition.*—Animals conditioned with the H-ion and those not conditioned gave almost identical head frequencies when allowed to reconstitute in well water, pH 7.5. When reconstitution took place in carbonate-free well water, pH 4.15, the conditioned animals showed a distinctly higher head frequency in *B*, *C*, and *D* pieces than non-conditioned animals in the same solution.

TABLE IX

HEAD FREQUENCY AND VIABILITY OF ANIMALS CONDITIONED TO CARBON DIOXIDE AND ALLOWED TO RECONSTITUTE FOR VARIOUS INTERVALS OF TIME IN THE SAME SOLUTION

	Head-Frequency Index				Percentage Living			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Well water (control)	97.9	44.5	21.6	20.8	98.0	98.0	100.0	100.0
T-1, well water, pH 7.5	100.0	58.8	41.2	30.4	100.0	100.0	100.0	100.0
T-2, CO_2 solution, pH 5.35, 24 hours only	100.0	88.6	70.4	70.8	90.0	88.0	92.0	74.0
T-3, CO_2 solution, pH 5.35, 48 hours only	100.0	90.9	73.2	79.4	96.0	100.0	82.0	68.0
T-4, CO_2 solution, pH 5.35, 72 hours only	100.0	86.5	65.1	59.6	100.0	98.0	94.0	92.0
T-5, CO_2 solution, pH 5.35, entire period	99.6	84.9	65.8	58.8	98.0	82.0	90.0	90.0

Worms conditioned with the H-ion and allowed to reconstitute in pH 4.15 plus 0.5 gm. of $CaCl_2$ per liter gave a very interesting result. Instead of calcium causing a decrease in head frequency, as it did in the case of non-conditioned worms, it actually caused an *increase* in head frequency at all four levels. In other words, in the case of H-ion conditioned worms, the head frequency in high concentrations of the H-ion with calcium was higher than in the same concentrations of H-ion without calcium.

Death of pieces in pH 4.15 was almost entirely prevented by conditioning the worms to H-ion prior to section.[3]

[3] Previous experiments with whole animals had given no visible evidence of an acquirement of tolerance to high concentrations of the H-ion. These experiments indicate,

3. *The effect of standing well water on physiological condition.*— Experiments comparable to those performed on worms conditioned to the H-ion and CO_2 were performed on worms conditioned to standing well water. It may be briefly stated that the head frequencies of worms conditioned in this manner were not substantially different from those of non-conditioned worms.

THE EFFECT OF THE HYDROGEN ION ON DIFFERENTIALS IN RATE OF EYESPOT FORMATION

It has been shown quite definitely throughout this investigation that there actually is a head-frequency gradient in *Euplanaria* and that this gradient can be altered by means of different reagents in the external medium. In this section we are not concerned with the head frequency except to point out the parallelism between it and another gradient, namely, the gradient in rate of eyespot formation. In speaking of the different gradients it is not meant to imply that there are different physiological gradients but that the different phenomena observed are different manifestations of the same axial differential.

Watanabe (1935*a*) has shown a gradient in rate of eyespot formation in this species when the different levels were allowed to reconstitute in well water. The object of the investigation here described is to determine whether external reagents can alter the eyespot gradient in a manner analogous to the alteration of the head-frequency gradient.

Animals in lots of 50 were sectioned in the manner shown in Figure 6. The section designated *X* comprises the anterior 5/16ths of the animal (exclusive of the head), while *Y* is the 5/16ths of the animal immediately posterior to *X*. Controls reconstituted in well water, pH 7.5, while the tests were submitted to carbonate-free well water, pH 4.20–4.15, for the reconstitutional period. The pieces were first examined at 36 hours after section. They were then examined every 12 hours until all showed definite eyespots. All examinations were made with the aid of a compound microscope. All the pieces of the size here described reconstituted into animals with normal heads.

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however, that there actually is some readjustment of the intact animals to pH. It appears from these data that the acquirement of tolerance for the H-ion is considerably less than for CO_2.

It is sometimes quite difficult, especially in *A* pieces, to tell with certainty the exact time of the first appearance of the eyespots. This is primarily due to the accumulation of pigment at the site of injury, which renders the minute eyespot invisible. Pieces recorded as having eyes at any particular time always showed the eyespots distinctly.

By recording the number of animals that had developed eyes during each 12-hour interval, it was possible to get a mean rate of eyespot formation with a standard error. From data obtained from the different levels under control and experimental conditions it was possible to demonstrate any differential in rate of eyespot formation, as well as any alterations which may have been caused by external factors.

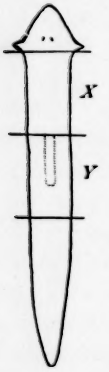

FIG. 6.—Sections *X* and *Y* (approximately 5/16ths of animal, exclusive of head) used in experiments on gradient in rate of eyespot formation.

The data for the rate of eyespot formation in pieces *X* and *Y*, as well as the head frequencies of 1/8th pieces (*A*, *B*, *C*, *D*, Fig. 1), from the same stocks of animals sectioned at the same time and allowed to reconstitute under the same test and control conditions are presented in Table X. In the first section of Table X are given the head-frequency indices. In the second section are given the rates of eyespot appearance in pieces *X* and *Y*. In the third section are given the differences in rate of eyespot appearance between (1) *X* and *Y* levels of both tests and controls ($Y-X$), (2) between *X* levels of tests and *X* levels of controls ($X_{II}-X_I$), (3) between *Y* levels of tests and *Y* levels of controls ($Y_{II}-Y_I$), and (4) between the differences found in (2) and (3) $[(X_{II}-X_I)-(Y_{II}-Y_I)]$.

The first experiment on rate of eyespot formation was performed in midwinter at a temperature of approximately 20° C. It will be noted (Lot 1, Table X) that the control worms showed a relatively high head frequency, while the differential in rate of eyespot formation between levels *X* and *Y* was quite small (3.6 ± 0.95 hours). In the test worms the head-frequency gradient had almost entirely dis-

appeared, and the eyespot gradient was of no significance (0.7 ± 1.43 hours). In comparing the rate of eyespot formation in the tests and controls at the same levels, it was found that the rate of development was definitely inhibited in high concentrations of the H-ion (10 ± 1.39 hours for X levels and 6.3 ± 1.14 hours for Y levels). It appears from these data that the rate of eyespot formation was inhibited to a

TABLE X

THE GRADIENT IN RATE OF EYESPOT FORMATION IN *Euplanaria dorotocephala* WITH ITS ALTERATION BY USE OF THE HYDROGEN ION

(Rate of eyespot appearance given in hours)

		Head-Frequency Index				Rates of Eyespot Appearance		Differences in Rate of Eyespot Appearance			
		A	*B*	*C*	*D*	*X*	*Y*	$Y-X$	$X_{II}-X_I$	$Y_{II}-Y_I$	$(X_{II}-X_I)-(Y_{II}-Y_I)$
Lot 1	I. Well water (control)	99	97	63	51	73.9 ± 0.82	77.5 ± 0.49	3.6 ± 0.95	10.6 ± 1.39	6.3 ± 1.14	4.3 ± 1.71
	II. Carbonate-free well water, pH 4.2	98	97	93	91	84.5 ± 0.98	83.8 ± 1.03	-0.7 ± 1.43			
Lot 2	I. Well water (control)	99	49	21	22	50.4 ± 0.86	64.1 ± 1.05	13.7 ± 1.35	9.4 ± 1.54	3.8 ± 1.56	5.6 ± 2.2
	II. Carbonate-free well water, pH 4.15	96	63	34	44	59.8 ± 1.28	67.9 ± 1.16	8.1 ± 1.73			

greater extent in X than in Y pieces (4.3 ± 1.71 hours). It seems to be quite clear that in the test solutions the physiological gradient may be flattened out or obliterated through differential inhibition.

The second experiment on rate of eyespot formation (Lot 2, Table X) was performed in the summer at a temperature of approximately 32° C. It will be noted that the control worms showed a very low head frequency, while the differential in rate of eyespot formation in pieces X and Y of similar worms was quite marked (13.7 ± 1.35 hours). The test worms (those which reconstituted in carbonate-free well water, pH 4.15) showed a higher head frequency in pieces B, C, and D and less differential in rate of eyespot formation

at levels *X* and *Y* (8.1 ± 1.73 hours). As in the case of the foregoing experiment, both levels are retarded in rate of eyespot formation (9.4 ± 1.54 hours for level *X* and 3.8 ± 1.56 hours for level *Y*). Here, again, level *X* is retarded more than level *Y* (5.6 ± 2.2 hours). In this later experiment the differential was so great between the different levels that it was only partially destroyed by the H-ion.

DISCUSSION

It has been pointed out repeatedly during this work that the inhibition of heads in short transverse pieces of *Euplanaria* is the result of some factor or factors arising from the posterior cut surface. This inhibiting influence may be present for several days and, as stated by Child and Watanabe (1935) and Watanabe (1935*b*), it exerts its effect by preventing in some way the dedifferentiation and activation of the head-forming cells in the anterior region of the piece. These authors have given evidence indicating that this inhibitory effect of posterior cut surface is transmitted forward from its point of origin chiefly by way of the ventral nerve cords.

It has also been found (Watanabe, 1935*a*) that a gradient in rate of head growth (eyespot formation) exists along the axis. This gradient of eyespot formation is apparently independent of any posterior effect, since it appears in long pieces in which head development is not inhibited. Those regions which show the slowest rate in eyespot formation are likewise the regions which give the largest number of inhibited head-forms with short transverse pieces.

It has been suggested (Child and Watanabe, 1935) that the inhibitory effect arising from the posterior cut surface may not come into play in transverse pieces from the more anterior levels because of a greater activity of the head-forming cells in these regions. It was also suggested that a decrement in the transmission of the inhibiting factor may be greater in the anterior levels and less in the posterior levels of the first zooid.

When anterior and posterior section are made at the same time in 1/8th *D* pieces, head frequency is low and acephalic forms predominate; while a delay in the posterior cut for 6–8 hours will result in the reconstitution of animals with 100 per cent heads, and with longer delay 100 per cent normal heads. From this it seems to be evident

that a short delay in bringing into action by posterior section the factor inhibiting head development is enough to permit at least a beginning in dedifferentiation and activation of the head-forming cells. With this advantage the anterior end is able to carry through the reconstitution of a normal head in spite of any inhibiting effect which later may be imposed from the posterior cut surface.

It seems quite clear that the increase in head frequency found in the experiments with CO_2 and H-ion is brought about through some kind of inhibition of the factor inhibiting head development. These agents may act directly in decreasing activity at the posterior cut surface which inhibits head formation, or they may act by penetrating and blocking any excitation passing forward from the posterior point of origin. It has been found in the experiments on rate of eyespot formation that carbonate-free well water with low pH (Table X) retards, to a certain extent, the whole reconstitutional process, while it increases head frequency. (Child, 1916, observed this same phenomenon with dilute solutions of KCN.) Because of this fact, it seems more logical to assume that the inhibition of the factor inhibiting head development is one aspect of a general inhibiting action which is more effective in decreasing or eliminating that factor, perhaps by block, than in inhibiting the head-forming cells.

The effects of carbon dioxide.—It has been seen from the experiments on head frequency that CO_2 and the H-ion behave quite differently in the alteration of head frequency even though the end results may be of like nature. It appears as if CO_2 penetrates and produces its inhibiting effects quite independently of external pH. It has been found (Osterhout and Dorcas, 1925) that in *Valonia* little or no CO_2 enters normal cells except in the form of undissociated molecules. Since HCl ionizes completely in the concentrations used and H_2CO_3 is only partly ionized, it seems probable that at least some of the differences in the effects observed are related to the differences in penetration of CO_2 and the H-ion. Hyman (1925) has found that CO_2 depresses considerably the oxygen consumption in *Euplanaria*. Haywood (1927), in describing CO_2 as a narcotic agent, has shown that high concentrations almost completely suppress cleavage in the eggs of *Arbacia*. Jacobs (1912, 1920*a*, 1920*b*, 1922) has shown that the action of CO_2 is apparently independent of the

H-ion and that H_2CO_3 differs from the strong acids in its ability to penetrate living cells. Its action has been described by this author as being more or less specific. He found that CO_2 produces first a liquefaction, followed by coagulation of the protoplasm of *Spirogyra*. Fox (1932, 1933*a*, 1933*b*) goes into the subject of CO_2 effects, particularly on the streaming of protoplasm, and attributes the phenomena observed to CO_2 narcosis rather than a H-ion effect. In considering the subject of CO_2 versus H-ion, Heilbrunn (1928, p. 188) points out that CO_2 in solution is more or less anesthetic in action, has a lower surface tension than the pure solvent, and may act as a fat solvent in decreasing protoplasmic viscosity.

Rustia (1925) found that HCl added to well water increased the number of bipolar forms arising from short transverse pieces of *Euplanaria*. He also observed an increase in head frequency with this treatment. As he did not remove the bicarbonates from the well water, it is probable that his results are due to CO_2 rather than the H-ion.

In the experiments with CO_2 on head frequency described in this paper, it has been shown that CO_2 is effective over a wide range of concentrations and that the effect does not depend on the external H-ion. It has also been shown that worms submitted gradually to high concentrations of CO_2 will acquire a tolerance to solutions toxic to worms not so treated. It appears, in view of the previous work done on CO_2, that all processes pertaining to growth are retarded but that the inhibitory factor arising from the posterior cut surface is affected to a greater extent than the head-forming cells. With this condition existing, it is clearly seen how short transverse pieces of *Euplanaria* which normally give a low head frequency may give increased head frequency in CO_2.

The effects of hydrogen ion.—In our experiments with carbonate-free water it has been possible to demonstrate the actual effect of H-ion on reconstitution in *Euplanaria*. It has been shown, in the experiments on rate of eyespot formation, that the H-ion in the proper concentration retards, to a measurable extent, reconstitutional development. In concentrations below a critical value (pH 4.3) there is apparently little or no visible effect of the H-ion, while in higher concentrations (pH 4.1 or below) death results. This effect of

the H-ion is doubtless due to the impermeability of living membranes to low concentrations of the ions of strong acids.

Osterhout (1914), in measuring the permeability of tissues of *Laminaria saccharina* by means of their electrical resistance, found that HCl produced a rapid decrease of permeability, which was shortly followed by a rapid increase, which continued until the death-point was reached. In the light of these and other experiments it appears probable that the H-ion does not enter the planarian tissue below a certain concentration (pH 4.3) because of permeability factors. Through a short range of higher concentrations the permeability of the tissues for the H-ion increases, and this results in an increase in the internal acidity. This fall of internal pH may result first in a decreased viscosity of the protoplasm (Heilbrunn, 1928). With a further decrease in pH, coagulation of the protoplasm occurs. It seems extremely likely that metabolic processes are affected by changes in internal pH.[4]

Jewell (1920), in her work on environment and regeneration, found that certain concentrations of HCl in distilled water retarded CO_2 production and regenerative growth in tadpoles. In Table 6 of her paper she records a pH of 5.4 as being inhibitory to these processes. She reports a concentration of N/0.00025 HCl as having a pH of 5.4; *N*/0.0005 HCl, a pH of 3.8; and *N*/0.00075 HCl, a pH of 3.2. From these figures it appears that some other factor besides pH is concerned, especially in solutions of pH 5.4. Hyman (1925) found that strong acids in carbonate-free well water had absolutely no effect on rate of oxygen consumption in planarians between pH 7.5 and 5.0. A slight depression (about 15 per cent) was found at pH 4.0. Heilbrunn (1928) suggests that the H-ion may liberate CO_2 from protoplasmic buffers and therefore act in much the same way as the direct penetration of CO_2. This suggestion is invalidated, at least in part, by Barth (1929), who shows that a number of strong and weak acids will produce coagulation in *Arbacia* eggs at the same external pH if the permeability factor is removed with NaCl. (At the same pH the different acids would be in different concentrations. The

[4] It is apparent that CO_2 also alters the internal pH, and indeed it seems probable that these pH changes may be equally as important as the narcotic action of CO_2 in altering reconstitutional development.

amount of CO_2 liberated depends on the concentration of the acid rather than on the pH.)

In the case of the H-ion it is apparent that the head frequency in *Euplanaria* is increased by a greater inhibition of the effect of the posterior cut than of the head-forming cells. It is also of much interest that the anterior levels of the first zooid are retarded in rate of growth to a greater extent than the more posterior levels of the same zooid. As the anterior levels are originally more active, this greater retardation is a good example of differential inhibition, a phenomenon that has been noted so often in this laboratory.

Calcium antagonism to the hydrogen ion.—It has been shown in these experiments that certain effective concentrations of the H-ion can be almost completely antagonized, as regards head frequency, by increasing the calcium concentration of the solution. It is known that calcium is used by living cells in the formation of such structures as the plasma membrane. Salts like sodium and potassium increase the permeability and break down cell membranes. The antagonistic action of calcium on sodium and potassium is too well known to need mentioning. It seems in these experiments that calcium antagonizes the activity of the H-ion by preventing the increase in permeability of the cell membranes which accompanies high concentrations of the H-ion. It is also possible that the antagonistic action of calcium on H-ion may be accomplished to some extent by the different actions of these agents on the protoplasmic colloids.

The experimental alteration of physiological condition.—It has been seen throughout these experiments that different lots of animals of the same size often have quite different head frequencies under the same experimental conditions. It appears probable that animals giving a high head frequency at the more posterior levels of the anterior zooid have less axial differential than those giving low head frequencies. However, since head frequency depends on two antagonistic factors (the capacity of the cells concerned in head formation to react to conditions resulting from anterior section, and the factor inhibiting activation and dedifferentiation of these cells which originates at the posterior cut surface), it is evident that the relation between the head-frequency gradient and the general axial differential is not simple and direct. The condition of the head-forming cells and

the effectiveness of the inhibiting factor may vary and can be altered experimentally in either direction, and head frequency in any given case depends on the relation between them. The differences in time of eyespot appearance at different body-levels (Table X) indicate, first, a graded difference in condition of the head-forming cells at different levels, which is apparently a direct expression of the general axial differential, and, second, variation in the amount of this difference in different stocks and under different experimental conditions, which suggests differences in steepness of the general gradient. It is not yet known, however, whether the factor inhibiting head development shows similar graded differences. In the light of the facts at present available, the head-frequency gradient appears to be an indirect and complex expression of the axial gradient involving two opposing and variable factors.

The object of the experiments on conditioning to CO_2 was to alter, if possible, the relation between these two factors, and so to alter head frequency. As is evident from Table IX and Figure 5, the head frequencies of conditioned animals are in all cases higher at the more posterior levels than in the non-conditioned; but there is no significant alteration in head frequency at anterior levels. It seems probable in the light of these results that the chief—perhaps the only—effect of the conditioning is the inhibition of the factor inhibiting head development at the more posterior levels. Since this inhibiting factor is apparently chiefly or wholly nervous in character, the conditioning in this case probably consists chiefly in the narcotic action of CO_2. The conditioned animals behave as if partially narcotized. They show decreased motor co-ordination and frequently undergo fission, as they do in other narcotics. Certainly the conditioning to CO_2 has little or no effect on the head-forming cells, even at the most susceptible anterior levels, for there is no decrease in head frequency there; and, since the inhibiting factor is not effective at anterior levels in 1/8th pieces, there is no increase. Obviously, head frequency can be altered at the more posterior levels by conditioning to CO_2 before sectioning, but the data indicate that the effect is chiefly on the nervous system rather than on the general axial gradient. The very great increase in head frequency in animals conditioned to CO_2 and subjected to H-ion after section (Fig. 5 *M'*) evidently results from the

combined effect of CO_2 in the protoplasm and the H-ion administered. The combined effect of the two agents may provide a more effective block against the inhibitory factor, but it does not necessarily follow that their effects are directly additive physiologically. If this were the case, an increase in percentage of deaths over the non-conditioned lot would be expected, since both CO_2 and H-ion are near the lethal concentration; but such increase does not appear.

The data on time of appearance of eyespots (Table X) suggests that some degree of leveling out of the general axial gradient does occur in the H-ion concentration used. The increase in time in H-ion is greater at the anterior level (X) than at the posterior level (Y) in both lots; and the difference in time between X and Y is less in the experimental than in the control lots. The pieces used in these experiments are long enough to give 100 per cent normal heads. Consequently, the times of appearance of eyespots are regarded as indicating differences in condition of the cells concerned in head formation at different levels.

SUMMARY

Euplanaria dorotocephala, 14–16 mm. in length, were cut into short transverse pieces (approximately 1/8th the length of the animal, exclusive of the head). Only the first four pieces (A, B, C, D), which make up most of the anterior zooid, were used. These pieces were caused to reconstitute under various experimental conditions. Upon reconstitution the head frequencies and the number dead at each level were recorded. All experimental lots were controlled with similar lots in well water.

1. Lots of reconstituted worms from animals taken directly from well water, sectioned, and permitted to reconstitute in CO_2 solution (pH 5.4–5.6) show an increased head frequency in levels B, C, and D. The death frequencies were greater in pieces A and D.

2. Intact animals submitted to high concentrations of CO_2 for a few days will become acclimated to CO_2 and be able to withstand considerably higher concentrations or concentrations which are lethal to animals that have not been previously treated with this reagent.

3. The effects of CO_2 are not due to the external pH, as experiments with carbonate-free well water show that the H-ion has little

or no effect on reconstitution in *Euplanaria* at the pH values of the CO_2 solution.

4. Head-frequency experiments in which pieces reconstituted in carbonate-free well water, pH 4.15–4.30, indicate that this concentration of the H-ion induces an increase in head frequency at levels *B*, *C*, and *D*. As in the case of CO_2, the death frequencies are greater in *A* and *D* pieces.

5. When $CaCl_2$ (0.5 gm. per liter) is added to carbonate-free well water, pH 4.15, the effect of the H-ion in increasing head frequency is almost completely antagonized. Animals are also protected from the lethal effects of the H-ion by the addition of calcium to the test solution.

6. Stocks of worms from different localities and different lots of worms from the same locality (collected at different times) may yield very different head frequencies under the same experimental conditions. Worms from the same locality and collected at the same time may vary as regards head frequency, depending on the physiological condition of the stock at the time of section and upon the length of time the worms have been kept in the laboratory.

7. Worms have been experimentally conditioned by submitting them to high concentrations of CO_2 and H-ion for several days before section. These conditioned worms react quite differently, as regards head frequency, than do non-conditioned worms from the same stock. The chief difference is an increased head frequency in the conditioned worms. Carbon dioxide is a much more effective agent for conditioning than H-ion.

8. In experiments on rate of eyespot formation at different body-levels in *Euplanaria* it was found that 5/16-*X* pieces (anterior 5/16ths of animal exclusive of the head) developed eyespots before 5/16-*Y* pieces (middle 5/16ths of animal). In carbonate-free well water (pH 4.15–4.20) the time of eyespot formation was longer at both levels, but *X* was retarded to a greater extent than *Y*.

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GONAD DIFFERENTIATION IN THE CHICK EMBRYO AS STUDIED IN HETEROSEXUAL GRAFT AND HOST-GRAFT COMBINATIONS[1]

(Two plates)

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LILLIE (1917) reported that the embryonic sex glands of the female member of a pair of opposite-sexed twin calves are modified in the male direction as the result of a fusion of its chorionic blood vessels with those of its male co-twin. Several investigators have since employed the chorioallantoic grafting technique as a means of incorporating two gonads of opposite sex in the same embryonic blood circulation of the chick embryo. Such a host-graft relationship would seem to be a suitable method of testing the influence of a gonad graft upon the reproductive organs of the host embryo, or of the gonads of the host upon the engrafted gonad.

Previous experiments involving the transplantation of gonad tissue to the chorioallantoic membrane have been of two general types: (1) Gonad rudiments were transplanted to host embryos whose sex glands were already morphologically differentiated to determine what influence the sex of the host might have upon the capacity of the rudiment to produce a gonad of specific sex (Willier, 1925 and 1927, and Corinaldesi, 1927*a* and 1927*b*). (2) Sexually differentiated gonad tissue isolated from embryos and from juvenile or adult fowl were transplanted to host embryos whose sex glands were in an early stage of morphological sex differentiation (7–9 days' incubation). The latter type of experiment was carried out by Minoura (1921), Greenwood (1925), Kemp (1925 and 1927), and Willier (1927) to determine whether or not the gonad graft influences the process of differentiation in the sex glands and ducts of the host. With the exception of those reported by Minoura, these experiments were negative on the question of sex hormone action in the chick.

[1] This work was carried out at the suggestion and under the direction of Dr. B. H. Willier, to whom I am greatly indebted for helpful suggestions and valuable aid.

Consequently, a further study, employing different methods of approach, seemed advisable.

The first series of experiments reported here was designed to test the self-differentiating capacity of the morphologically undifferentiated gonad when associated for 8 or 9 days in the same chorioallantoic graft with differentiated gonad tissue (isolated from birds ranging in age between 12 days' incubation and $3\frac{1}{2}$ months' posthatching). This type of experiment enables one to test possible effects of contiguous or closely associated cortical and testicular tissues upon the direction of differentiation of the indifferent gonad rudiment. Such a relationship may be of primary importance in influencing the course of sex differentiation, as Witschi (1931 and 1934) has suggested for amphibians.

Certain combination grafts in this series contain gonad tissue known to be producing hormone at the time of implantation, i.e., from hatched chicks. Such grafts should throw some light on the question of the capacity of sex hormones which condition secondary sexual characteristics to function also as sex-differentiating hormones.

The second group of experiments was performed to ascertain what influence gonads, already structurally differentiated as to sex, and of the same age, may exert upon each other in heterosexual combination in the same graft. Testes and ovaries were isolated from donors of approximately 18 days of incubation and transplanted in heterosexual pairs to a host embryo, where they developed in close proximity or in direct contact with one another for a period of 8 or 9 days.

The third set of experiments was performed in an endeavor to elicit a response on the part of the reproductive organs of the host embryo to large amounts of gonad tissue transplanted to the chorioallantoic membrane. So-called "multiple-testis" grafts were made in an attempt to effect in the host embryo a high dosage of male hormone, thereby possibly inducing modification of its reproductive organs by increasing the stimulus above a certain threshold.

MATERIAL AND METHODS

Donor material composing the grafts consisted of: (1) chick gonads morphologically undifferentiated as to sex, and (2) morphologically differentiated gonad tissue. The undifferentiated gonad

rudiments were obtained by isolating separately the right and left urinogenital ridges from embryos incubated between $4\frac{1}{2}$ and 6 days. This operation was performed under sterile conditions in a Petri dish containing warm physiological saline. The differentiated gonad tissue was secured from embryos during the latter half of their incubation period or from hatched birds as old as $3\frac{1}{2}$ months. Immediately after removal, this tissue was placed into warm, sterile salt solution. The grafting technique was essentially the same as that described by Willier (1924 and 1927). The tissue or tissues chosen to compose the graft were removed from the salt solution on the point of a glass needle and placed at the junction of two blood vessels on the chorioallantoic membrane of a previously prepared 8- or 9-day host embryo.

When a right gonad rudiment was combined with differentiated gonad tissue in a combination graft, the left one was transplanted alone to the membrane of a second host, thus serving as control; and vice versa. According to Willier (1927), the morphologically indifferent gonad has the capacity to self-differentiate according to its original plan of organization regardless of the sex of its host. Such a control graft thus affords a means of ascertaining the originally determined sex of its partner in the combination graft.

Eight or 9 days after operation the grafts were removed, fixed in Bouin's fluid, sectioned at 7 μ and stained with iron haematoxylin. The reproductive organs of all hosts were closely examined under a binocular microscope for evidence of any atypical structure. The ovaries of female hosts bearing "multiple-testis" grafts were examined histologically. A number of control grafts of testicular or ovarian tissue, as well as normal ovaries and testes of an age corresponding to those contained in the combination grafts, were frequently referred to during the examination of the experimental material. Eggs and tissues from the white Leghorn breed of fowl were used exclusively in these experiments.

ASSOCIATION OF AN INDIFFERENT GONAD RUDIMENT WITH DIFFERENTIATED GONAD TISSUE

A. THEORETICAL NUMBER OF GONAD COMBINATIONS

At the time the structurally indifferent gonad primordium (4–6 days' incubation) is transplanted with older testicular or ovarian tissue, only its age and laterality are known. During the 8 or 9 days

on the membrane the rudiment undergoes sex differentiation. To test its self-differentiating capacity in the presence of older gonad tissue, eight types of double transplants were made: right or left gonad rudiments, structurally undifferentiated as to sex, were transplanted with a piece of a right or left ovary or a right or left testis. The control in each case was the opposite gonad rudiment transplanted alone to a second host.

If the structurally undifferentiated gonad has the capacity to self-differentiate in accordance with its originally determined sex, and the laterality of each component of the graft is considered, sixteen possible combinations of gonad tissue should arise from the eight types of double transplants. Considering the marked and significant difference in structure and function between the left and the right ovary in the domestic fowl, it was essential to record the laterality of all ovarian tissue transplanted. Since, on the other hand, no such differences exist between the right and left testis in late embryonic, juvenile, or adult birds, the number of possible gonad combination may be reduced to twelve if the laterality of the testicular tissue transplanted is disregarded.

If the sex of the host is considered, twenty-four possible gonad relationships exist, twenty-three of which have been obtained in these experiments. It was found that each of the twelve possible gonad combinations was represented in the combination grafts, following the sex differentiation of the structurally indifferent component. In a number of cases the gonad rudiment which served as a control to its partner in a combination graft was lost. This is to be expected, since death of the host frequently follows the operation. In eighty-three out of the total one hundred and twenty-three cases the gonad rudiment in both the combination and the control graft survived. Table I shows the twelve possible types of combination graft, the number of cases recovered representing each type, and their relation to the sex of the host. Those in which the gonad rudiment gave rise to a gonad-like body, unspecific as to sex, are also recorded.

B. THE MORPHOLOGY OF THE "INDIFFERENT" GONAD PRIMORDIUM AT THE TIME OF IMPLANTATION

The majority of gonad primordia transplanted in combination grafts were isolated from donor embryos which had been incubated

for 5 or 5½ days. Two were as old as 6 days; fourteen were 4 and a fraction days of age.

At the end of the fourth day of incubation, according to Swift (1915), the developing gonad consists of a whitish ridge approximately 1.5 mm. in length lying on the ventromedial surface of the Wolffian body. It is composed of three types of tissue: (1) a germinal epithelium, two or three cell layers in thickness; (2) a compact mass of embryonic mesenchyme which lies beneath the germinal

TABLE I

Associated Differentiated Gonad	Sex of Host	Right Undifferentiated Gonad			Left Undifferentiated Gonad			
		Right Ovary	Right Testis	Right Not Ascertained	Left Ovary		Left Testis	Left Not Ascertained
					Typical	Atypical		
Left ovary	♀	3	2	1	4	1	2	0
	♂	2	2	0	1	0	4	1
Right ovary	♀	3	1	0	4	0	5	0
	♂	2	0	1	1	3	5	1
Testis	♀	1	4	0	7	1	14	4
	♂	4	2	3	4	14	11	5
Total	.....	15	11	5	21	19	41	11

epithelium; and (3) primordial germ cells, which are found between the epithelial cells of the germinal epithelium, as well as lying deeper in the embryonic mesenchyme. It is an interesting fact that the number of primordial germ cells at this early stage of development is greater in the left gonad rudiment than in the right (Firket, 1914; Swift, 1915).

At approximately the 132d hour of incubation the primary sex cords (cords of first proliferation) begin to invaginate into the underlying mesenchyme from the germinal epithelium. This process continues for the next 24 hours, certain of the sex cords in the meantime making connection with certain Malpigian corpuscles of the Wolffian body by means of the rete cords (cords of urinogenital union). At 6½ days (156 hours) the proliferation of the primary sex cords rather

abruptly ceases and sexual differences become morphologically apparent.

In these experiments, therefore, the gonad primordia contributing to the combination grafts or transplanted singly as controls were at the time of implantation "morphologically indifferent" as to sex.

C. SEX OF GONADS DIFFERENTIATING FROM GRAFTED RUDIMENTS

At 14 or 15 days of incubation the gonad of the chick has a characteristic histological structure which enables one to determine the sex, and, in the case of the female, the laterality. With certain exceptions, which will be described later, the gonad rudiments which gave rise to gonads in combination grafts possess a similar characteristic histological structure.

The typical left ovary found in a combination graft has two markedly different structural components. It possesses both a cortex and a medulla (Plate I, Fig. 3). The medulla is composed of a diffuse network of epithelial cords. The cortex is more compact in appearance and is made up of cords of epithelial and germ cells which have invaginated from the germinal epithelium, at this time still composed of columnar epithelial cells and a few germ cells. This cortical layer overlies the medulla and covers the entire surface of the ovary with the exception of the area where the gland is attached to the Wolffian body. In short, the appearance of the ovary is strikingly similar to a normal left ovary of corresponding age.

The right ovary found in a combination graft differs from the typical left ovary in three principal characteristics: (1) its much smaller size; (2) absence of a set of cortical cords; and (3) a flat, inactive epithelial covering (Plate I, Fig. 1).

A testis which arises from a gonad rudiment in a combination graft can be easily distinguished from either the left or the right ovary since it possesses a well-developed tunica albuginea and a set of elongated sex cords whose structural components—the sex cells and supporting epithelial cells—are arranged in a characteristic spacial relationship within the cord (Plate I, Fig. 2). The identification of sex of all gonads arising from transplanted "indifferent" gonad rudiments was made on the basis of the above-mentioned criteria.

PLATE I

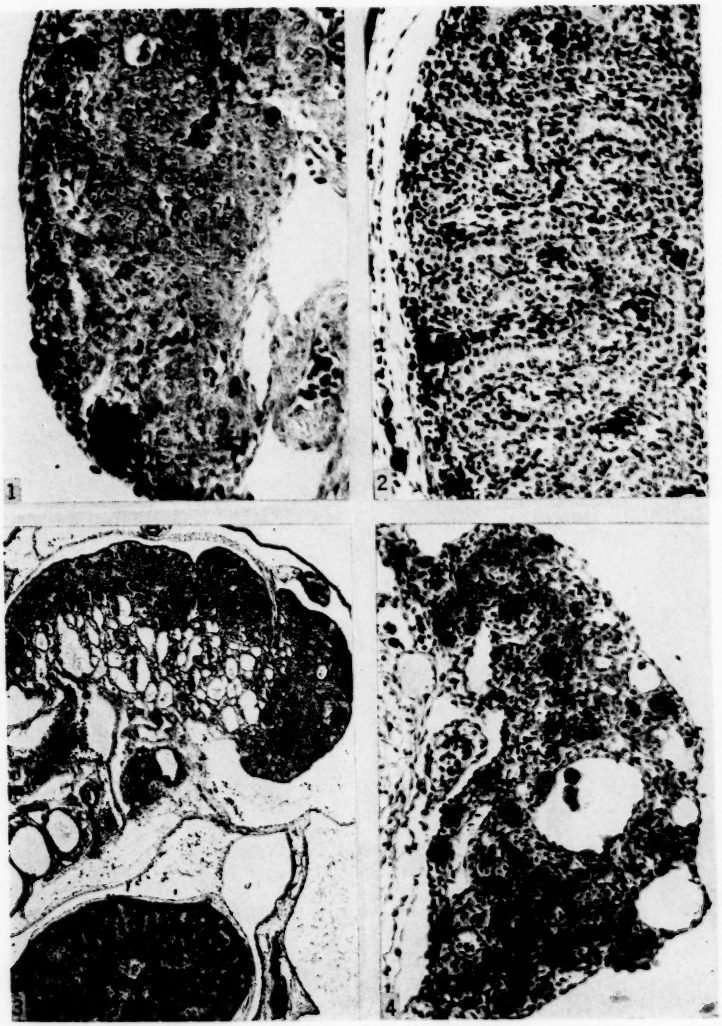
FIG. 1.—Section through a right ovary which differentiated from a right gonad rudiment combined with testicular tissue from a 51-day-old cock; grown for 9 days on a male host. ×500.

FIG. 2.—Section through a testis derived from a right gonad rudiment combined with a left ovary from an embryo of 12½ days' incubation and grown on a female host. ×400.

FIG. 3.—Longitudinal section through a left ovary differentiated from a left gonad rudiment in combination with a 17-day embryonic testis; 9 days on a male host. Note well-differentiated medulla and cortex. ×65.

FIG. 4.—Section of a modified left ovary derived from a left gonad rudiment, combined with an 18-day right ovary and grown on a male host. ×315.

PLATE I



The older gonad tissue which was transplanted with the sexually undifferentiated rudiment is strikingly different from the young sex gland which has undergone differentiation during the growth period of the graft. The difference in age is associated with sharp histological differences in structure, thus facilitating identification of the two components. Even in those cases where the sex of the younger gonad and the older gonad tissue is the same, no opportunity for confusing the two exists.

D. DIFFERENTIATION OF THE RIGHT "INDIFFERENT" GONAD PRIMORDIUM

Thirty-one grafts were examined in which a right "indifferent" gonad rudiment was combined with ovarian or testicular tissue. Fifteen differentiated into right ovaries, eleven into testes, and five into gonad-like bodies whose sex could not be ascertained (see Table I).

1. *Right ovaries.*—Five right ovaries differentiated in grafts with left ovarian tissue (from embryos between 13 and 19 days' incubation), three of which developed on female and two on male hosts. Five were combined with right ovaries from embryos of 18 days' incubation, three of which developed on female and two on male hosts. Of the remaining five, which developed with testicular tissue from chicks ranging in age between 17 days' incubation and 80 days' post-hatching, four developed on male, and one on a female, host.

Regardless of the nature of the combination, the right ovary, which differentiates from the right indifferent gonad, is small and quite similar to the normal in general appearance. In the greater number of cases it consists of medullary elements only, although occasionally it is found to possess a few scattered areas of cortical tissue. That the presence of these cortical elements is not due to any specific factor of the environment operating during the development of the graft is strongly suggested by their rather frequent appearance in normal right ovaries (Brode, 1928).

In the majority of cases the right ovary is a more or less compact, definitely circumscribed, elongated body, broadly attached to the mesonephros (Plate I, Fig. 1). Occasionally, however, it may have a loose texture and present the appearance of a stromal network. Histologically, it consists of cords of epithelial or primordial germ

cells imbedded in a connective tissue stroma. Near the point where the ovary is attached to the Wolffian body, distention of the medullary cords frequently occurs. In the lumina of such distended cords epithelial cells or primordial germ cells may be found, either singly or in nests consisting of several cells. The surface of the right ovary lacks a distinct germinal epithelium and is covered by a single layer of flattened cells. Even in those right ovaries in which scattered cortical elements are present, the overlying germinal epithelium is flattened and inactive. This inactive condition of the germinal epithelium is also characteristic of the normal right ovary (Firket, 1920).

On theoretical grounds it is expected that the right ovary, known to possess male potentialities, might be modified in the male direction under the stimulation of hormones from older testicular tissue. In five cases right ovaries differentiated from right gonad rudiments which grew in the same graft with testicular tissue obtained from donors ranging in age from 17 days' incubation to 80 days' post-hatching. Four of these grafts grew on male hosts; one, on a female host. That these gonad rudiments were really determined as right ovaries is shown by the fact that, in the three cases in which the partner left gonad rudiment survived, it differentiated into a left ovary. Although there are slight variations in the structure of the right ovaries, they are within the range found characteristic for the normal right ovary. Furthermore, they differentiate equally well when combined with a testis and grown on a male host as when combined with ovarian tissue and grown on a female host.

2. *Right testes.*—Four of the eleven testes which differentiated from right gonad rudiments were combined with left ovarian tissue from donors ranging in age between 13 days' incubation and 75 days' post-hatching. Two grew on male, and two on female, hosts. One testis was combined with a right ovary from an embryo of 17½ days' incubation and grew on a female host, and the remaining six were associated with testicular tissue from embryos of 18 days' incubation or from chicks as old as 80 days. Four of these developed on female, and two on male, hosts.

Testes which arise from right gonad rudiments in combination grafts are similar in structure to those arising in grafts of right rudi-

ments transplanted alone, and closely resemble those described by Willier (1927). Their essential structural components consist of a surrounding tunica albuginea which is continuous with the connective tissue stroma containing the sexual cords. These cords are composed of supporting epithelial cells and primordial germ cells.

3. *Right gonad-like bodies.*—Although the right gonad rudiment differentiates in the majority of combination grafts into gonads of specific sex, in five such grafts it gave rise to gonad-like bodies whose sex could not be definitely ascertained. These vary considerably in structure. They are always small and generally poorly organized. Some have a definite shape and are rather sharply delimited from other structures in the graft, in which case they present a compact appearance and consist of "nests" of epithelial cells and germ cells imbedded in supporting connective tissue. Others can be considered little more than scattered groups of epithelial and primordial germ cells which occur in the mesenchyme of the graft in the vicinity of the mesonephric body.

It is necessary to investigate the conditions under which these right gonad-like bodies develop, to determine whether their origin may be related to the sex of the associated gonad or to the sex of the host.

Of the five, one was combined with a right ovary from an embryo of 15 days' incubation and grown on a male host; three with a pair of testes from 16-day embryos, on male hosts; and one with left ovarian tissue from an 80-day-old hen on a female host. Unfortunately, four of the five left gonad rudiments which served as controls for these grafts were lost, because of death of the host, and the originally determined sex of four of the five right rudiments in the combination grafts was therefore not ascertained. In the fifth case the right rudiment, which failed to produce a gonad of specific sex, had a female-determining constitution. It was associated with two testes from a 16-day embryo and grew on a male host. Little direct evidence is available, therefore, which points to a positive correlation between the sex of the associated gonad and the failure of the rudiment to differentiate specifically as to sex. Certain indirect evidence, however, indicates that the origin of these right gonad-like bodies is not due to sex hormones secreted by either the associated gonad or the

sex glands of the host. (1) Right gonad rudiments, originally determined female, develop into typical right ovaries when associated in the same graft with testicular tissue and grown on male hosts (see Plate I, Fig. 1). (2) Testes having a normal appearance arise from right gonad rudiments transplanted with left ovarian tissue and grown on female hosts (see Plate I, Fig. 2). (3) Four morphologically "indifferent" right gonads which were transplanted alone as controls developed into gonad-like bodies of undetermined sex, similar in structure to those occurring in the combination grafts. Of these four cases, two developed on male, and two on female, hosts.

E. DIFFERENTIATION OF THE LEFT "INDIFFERENT" GONAD

Ninety-two grafts were obtained in which a morphologically undifferentiated left gonad developed in combination with older ovarian or testicular tissue. Forty-one differentiated into left testes and forty into left ovaries. The remaining eleven gave rise to gonad-like structures which are undistinguishable as to sex.

1. *Left testes.*—Of the forty-one testes, six were combined with left ovarian tissue obtained from donors ranging in age between 18 days' incubation and 80 days' post-hatching. Two grew on female, and four on male, hosts. Ten were associated with right ovaries from embryos incubated $12\frac{1}{2}$–18 days. Of these, five developed on female, and five on male, hosts. Twenty-five developed in grafts with testicular tissue from donors ranging in age between $12\frac{1}{2}$ days' incubation and 51 days' post-hatching. Of these, fourteen grew on female hosts, the remaining eleven on male hosts.

Twenty-five of the forty-one right rudiments which served as controls to the left rudiments in combination grafts survived, each of which differentiated into a testis, thus proving that their partners in the combination grafts differentiated according to their originally determined male constitution.

The testes derived from left gonad primordia in combination grafts are similar in every respect to those already described which differentiated from right gonad rudiments grown under similar conditions. Moreover, they are identical in histological structure to those arising from either left or right gonad rudiments which were transplanted alone to the chorioallantoic membrane.

2. *Left ovaries.*—Left gonad rudiments, whose originally determined sex is female, exhibit wide variation in their capacity for self-differentiation in combination grafts. Of the forty left ovaries, twenty-one are structurally similar to the normal left ovary of a 13- or 14-day chick embryo, and nineteen show structural abnormalities in varying degrees.

Of the twenty-one which show a typical left ovarian structure, five developed in grafts with older left ovarian tissue obtained from embryos of 14–18 days' incubation and from hatched fowl as old as 112 days. Four such grafts developed on female, and one on a male, host. Five developed in combination with right ovaries from 13- to 18-day chick embryos; four on female, and one on a male, host. Eleven developed in grafts with testicular tissue (donors ranging in age from 16 days' incubation to 48 days after hatching), seven of which developed on female, and four on male, hosts.

In these twenty-one cases the ovary is composed of well-differentiated cortical and medullary elements (Plate I, Fig. 3), both of which contain epithelial and germ cells. A few cortical cords are in the process of invagination and remain attached to the well-developed columnar germinal epithelium, while others have lost connection with their point of origin. The medulla shows an outer compact area and an inner area composed of much-distended cords. Such grafted left ovaries have been described in detail by Willier (1927). The histological picture is remarkably similar to that of a normal left ovary of the same age.

Although left ovaries closely resembling the normal may develop from left "indifferent" gonads in combination grafts, nineteen left ovaries developing from left gonad rudiments in similar grafts show rather striking deviations from the normal structure.

Of these nineteen, one was associated with left ovarian tissue from an 80-day-old hen and grown on a female host. Three were combined with right ovaries from 18-day embryos and grown on male hosts. The remaining fifteen were combined with testicular tissue from donors ranging in age between 16 days' incubation and 48 days' post-hatching. Fourteen of these developed on male, and one on a female, host. Sixteen of the nineteen control right gonads survived and differentiated into right ovaries, thus proving that their

partner left rudiments in the combination grafts were originally determined as female. These atypically formed left female sex glands have certain characteristics in common: (1) small size; (2) absence of a columnar-celled germinal epithelium; (3) reduction or absence of cortical cords; and (4) compact masses of cellular cords, apparently medullary, which contain a greater number of germ cells than is characteristic of the medulla of normal left ovaries of corresponding age. In general, their appearance is similar to that of right ovaries derived from the opposite right "indifferent" gonad transplanted alone as a control.

The atypical left ovaries show individual variation in structure, and therefore a number of them will be described separately. One which was combined with left ovarian tissue from an 80-day-old hen and grown on a female host shows, through four or five serial sections, a germinal epithelium composed of columnar cells from which one or two cortical cords have invaginated. The greater portion of its surface, however, is devoid of a typical germinal epithelium and is covered by a layer of flattened epithelial cells beneath which no cortical elements have been formed. The medulla, which composes nearly the entire gland, consists of a connective tissue stroma in which are imbedded the medullary cords. Primordial germ cells are abundant, typically arranged in "nests." That the gonad's originally determined sex was female is proved by the fact that its partner right gonad, transplanted alone, developed into a typical right ovary.

One of the three modified left ovaries which were combined with right ovaries obtained from chicks near hatching and grown on male hosts is illustrated in Plate I, Figure 4. The germinal epithelium is composed of flattened cells, and cortical cords are completely lacking. Many primordial germ cells, arranged in "nests," are present. Two or three open spaces, bounded by a layer of flattened epithelial cells, suggest a distention of medullary cords. In some of these open spaces are found primordial germ cells, either singly or in small groups. The ovary has, in general, a compact appearance and is found to consist apparently of medullary elements only. Unfortunately, the control right gonad was lost, owing to death of the host following operation. However, its structure so closely corresponds to other

atypical left ovaries that little doubt exists that the left rudiment which gave rise to it was originally determined female.

Of the fifteen atypical left ovaries which developed in combination with testicular tissue, three may be described to illustrate the range of variation in structure which they exhibit. A left gonad rudiment combined with a testis from an 18-day embryo and grown on a male host gave rise to the left ovary illustrated in Plate II, Figure 5. The gland is small and covered by a distinct germinal epithelium composed of cuboidal epithelial cells and a few germ cells. From this germinal epithelium several cellular cords have invaginated which are probably cortical cords, although they are more slender and contain fewer well-differentiated germ cells than do normal cortical cords. The deeper portion of the gland is composed of nests of cells which contain both epithelial and primordial germ cells. These structures probably represent the medullary component of the ovary.

The left female sex gland, shown in Plate II, Figure 6, developed in a graft with two testes obtained from a 17-day embryo. It was grown on a male host. It has a germinal epithelium covering only about one-third of its free surface, from which no cortical cords have invaginated. The remainder of the gland is comprised of medullary cords imbedded in a connective-tissue stroma. These medullary cords are made up of epithelial cells and a few primordial germ cells.

A left ovary which is extremely abnormal in structure was derived from an "indifferent" left gonad primordium combined with a testis from an 18-day chick embryo and grown on a male host (Plate II, Fig. 7). It is small and shows a germinal epithelium composed of flattened cells; no cortical cords are present. The gland is composed entirely of medullary cords which are made up of two types of cells, epithelial and primordial germ cells, the latter greatly predominating. This fact gives the gland the appearance, when examined under low magnification, of being composed almost entirely of "nests" of primordial germ cells. No distention of the medullary cords occurs in this left ovary. The control graft contains a well-differentiated right ovary.

It is evident, then, that left ovaries arising from "indifferent" left gonad rudiments combined with testicular tissue may appear quite

PLATE II

FIG. 5.—Section of a slightly modified left ovary which developed in the same graft with an 18-day embryonic testis. The host was a male. ×500.

FIG. 6.—Section of a modified left ovary derived from a left gonad rudiment which was associated with two testes obtained from a 17-day embryo; male host. × 525.

FIG. 7.—Section of an abnormal left ovary which developed from a left rudiment combined with an 18-day testis and grown on a male host. ×500.

FIG. 8.—Section of a gonad-like body possessing male sex cords and a germinal epithelium. It arose from a left gonad rudiment combined with a right ovary of a 16-day embryo. The host was a male. ×525.

PLATE II

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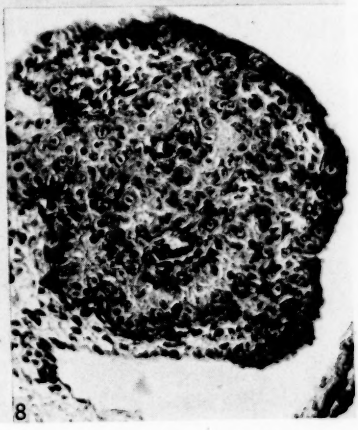
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normal or may show structural modification in varying degrees. In those less strikingly atypical in structure a germinal epithelium may be present from which a very sparse proliferation of cortical cords has occurred (one case); those showing greater modification may possess a germinal epithelium consisting of columnar or cuboidal cells but no cortical cords (two cases); finally, the left ovary may consist of medullary elements only, both germinal epithelium and cortical cords being entirely lacking (twelve cases). This tendency for left gonad rudiments having a female-determining constitution to develop in an atypical manner in combination grafts stands out in marked contrast to the capacity for typical differentiation exhibited by right rudiments which were originally determined female and by rudiments having a male-determining constitution (either right or left). It will be noted in Table I that, of the seventeen left ovaries which differentiated in combination grafts grown on female hosts, fifteen possess a typical structure and two are atypical. Of the twenty-three left ovaries in combination grafts supported by male hosts, only six are typical in structure, while seventeen present an atypical histological picture. These data, examined statistically, give a difference of 62.1 per cent, or 3.88 times the standard error. Only 12.5 per cent of the left ovaries which differentiated in grafts containing testicular tissue and which grew on female hosts were abnormal, while similar left ovaries also associated with testicular tissue but grown on male hosts present an atypical histological picture in 77.7 per cent of the cases. It would appear, therefore, that even though the sex of the differentiated gonad tissue in the same graft is disregarded, the sex of the host must be considered a possible factor influencing the capacity of the left rudiment, originally determined female, to differentiate into a typical left ovary.

3. *Left gonad-like bodies.*—Of the eleven gonad-like bodies derived from left gonad rudiments, one was combined with a left ovary from a 19-day embryo and grown on a male host. Another developed, with a 16-day embryonic right ovary, on a male host; and nine were found in grafts with testicular tissue from donors ranging in age between 14 days' incubation and 80 days' post-hatching. Of these nine, four developed on female, and five on male, hosts.

With one exception, these gonad-like bodies are very similar in structure to those which develop from right gonad rudiments. The one exceptional left gonad, which developed in a graft with a right ovary, on a male host, has definite structural peculiarities (Plate II, Fig. 8). Most of its free surface is covered with a germinal epithelium composed of columnar cells between which occur primordial germ cells. The greater portion of the gland is composed of well-differentiated sex cords which closely resemble, structurally, those of a normal testis of a chick of approximately 14 days' incubation. The primordial germ cells are distributed irregularly throughout the sex cords, while the supporting epithelial cells are arranged radially around the long axis of the cord, their oval nuclei showing a linear arrangement along the periphery. Although the sexual cords are typically testicular in appearance, the germinal epithelium is more characteristic of a left ovary. Unfortunately, the control graft containing the partner right gonad was not recovered.

The originally determined sex of four of these eleven gonad-like bodies was ascertained. These four, originally determined male, were combined with testicular tissue from donors aged 50 days after hatching. Two of them developed on female, and two on male, hosts.

Three similar gonad-like bodies developed from left gonads transplanted alone. Two of these grafts developed on female, the remaining one on a male, host.

F. DEVELOPMENT OF THE OLDER GONAD COMPONENT IN THE GRAFT

Testes isolated from chicks just before, or a few days after, hatching become completely vascularized following transplantation, and continue to develop in essentially a normal manner (Plate I, Fig. 3). Large transplants of testicular tissue from juvenile fowl (40–90 days of age) frequently fail to become completely vascularized rapidly enough to prevent necrosis at their center. Even in those cases where degenerative changes are evident centrally, a large number of apparently healthy and well-differentiated semeniferous tubules are present around the periphery of the transplant.

All right ovaries contributing to combination grafts were isolated from chicks during the last 4 or 5 days of the incubation period. After 8 or 9 days in the graft they are similar in appearance to nor-

mal right ovaries of chicks 4 or 5 days after hatching. They vary in size and shape, but their histological structure remains quite typically right ovarian.

Left ovarian tissue from embryonic or newly hatched chicks becomes rapidly vascularized following transplantation. Donor tissue isolated at 17 or 18 days of incubation shows well-developed Graffian follicles after 9 days on the host. Large pieces of ovarian tissue from young hens (60–112 days of age) may become necrotic centrally, owing to slow and incomplete vascularization following transplantation. The periphery of the transplant always shows many follicles containing ova, some of which have a massive accumulation of yolk, and nuclei containing formed chromosomes.

It can be definitely stated that gonad tissue from late embryonic or hatched fowl, when incorporated into a combination graft, continues to develop in a normal manner, with the exception of the central area of large transplants, which become vascularized too slowly to prevent degenerative changes.

COMBINATION GRAFTS OF SEXUALLY DIFFERENTIATED GONADS

To ascertain what influence gonads, already structurally differentiated as to sex, and of the same age, may exert upon each other in heterosexual combination in the same graft, testes and ovaries were isolated from donors of 18 days' incubation and transplanted in heterosexual pairs to a host embryo. There they developed in close proximity or in direct contact with one another for a period of 8 or 9 days.

A. COMBINATION GRAFTS OF TESTIS AND RIGHT OVARY

Seven grafts which resulted from a combination of a testis and right ovary show both gonads to have a structure essentially similar to normal gonads of corresponding age. The testis in each case continued to develop in a normal manner and exhibits a histological picture similar in most respects to the testis of a 5- or 6-day hatched chick. The semeniferous tubules are greatly elongated; and lumina, never present at the time of isolation, have developed in many of them. The right ovaries vary somewhat in structure, but not beyond the range of variation to which normal right ovaries are subject. In three cases the right ovary is a compact, definitely circumscribed

body. In two grafts it has the appearance of a network, and in one case has become infiltrated with lymphocytes. In another graft the right ovary is abnormally large and contains a considerable amount of cortical tissue. The protocol reveals, however, that areas of cortical elements were observed macroscopically on the surface of the ovary at the time of implantation.

B. COMBINATION GRAFTS OF TESTIS AND LEFT OVARY (NINE CASES)

Six grafts arising from the transplantation of left ovary with testis show both components of the graft to have continued their growth and development with little or no variation from their normal structure. In four of these six cases the testis and ovary are in direct contact, and probably had been so during the 9-day growth period on the host embryo. In the remaining three grafts the testis is quite normal in appearance and closely corresponds to the testis of a chick 5 or 6 days after hatching, but the associated left ovaries show an appreciable thinning of the cortical layer with a reduction in the number of primitive ova contained. Grafts containing normal left ovaries in the presence of testicular tissue are found on both male and female hosts. Of the three combination grafts showing ovaries with reduced cortex, two grew on male hosts and one on a female host. In two instances left ovaries of corresponding age transplanted alone on hosts (one male, one female) have shown a similar thinning of the cortical layer.

RESPONSE OF THE REPRODUCTIVE ORGANS OF THE HOST TO GONAD GRAFTS

The reproductive organs of all host embryos bearing combination, as well as control, grafts were examined under a binocular dissecting microscope for evidence of a reaction to the various types of gonad grafts. Some variations from the typical anatomical structure were observed in the reproductive organs of the hosts. These include variations in the size and shape of the gonads themselves, particularly the right ovary. The length of the persistent "cloacal stump" of the right oviduct and the diameter of the Wolffian ducts, the left oviduct, and of the shell gland are all subject to slight variations. These variations from the typical structure cannot be considered as

specific responses to gonad grafts, since they are very similar to those reported previously by Willier and Yuh (1928), who proved that they also occurred in host embryos bearing non-gonad grafts. Similar variations were observed in the structure of the reproductive organs of 18-day normally incubated chick embryos whose gonads served as donor tissue in these experiments.

"MULTIPLE-GONAD" GRAFTS

It seemed possible that lack of response to gonad grafts on the part of the hosts' reproductive organs might be due solely to the failure of the small pieces of transplanted gonad tissue to secrete sufficient sex hormone to bring about a positive response. It was hoped that grafts consisting of four or five 18-day embryonic gonads transplanted to 9-day host embryos might produce sufficient hormone to elicit a response in the reproductive organs of the hosts. Grafts consisting of as many as five testes from 18-day embryos developed for 8 or 9 days on both male and female hosts. The grafts were well vascularized, and the transplanted testicular tissue was apparently in a healthy and normal condition. The reproductive organs of the male hosts bearing such grafts were examined under the binocular dissecting microscope and found to correspond closely to those of a normally incubated 18-day male chick embryo. The sex ducts of the female hosts were normal in appearance, and histological examination of the ovaries of these hosts revealed no deviations from the normal structure. It would seem definitely established, therefore, that even large amounts of testicular tissue, from embryos just prior to hatching, are unable, when transplanted to the chorioallantoic membrane at 9 days' incubation, to affect the reproductive organs of the host.

No grafts resulting from the transplantation of three or more 18-day left ovaries were recovered, since the hosts in all such cases failed to survive the operation. Death of the host under these conditions was probably due to the large volume of tissue transplanted.

DISCUSSION

The evidence presented in the foregoing sections reveals that, with certain exceptions to be noted below, right or left indifferent gonad

rudiments, in case they have a male-determining constitution, have the capacity for self-differentiation into a testis regardless of the sex of the associated differentiated gonad and of the sex of the host. Either right or left gonad rudiments whose originally determined sex was male differentiated into testes although they were subject to hormones from an associated ovary (left or right) and grown on a female host.

Such a capacity for independent differentiation is likewise characteristic of a right gonad rudiment when it has a female-determining constitution. Such a right rudiment, although associated with testicular tissue of various ages and grown on a male host, has the capacity to differentiate into a typical right ovary.

The failure of the right ovary, developing under these conditions, to show any evidence of inversion is interesting, since it is known to have male potentialities and the capacity to transform into a testis following left ovariectomy of chicks just after hatching (Benoit, 1923*a*, 1923*b*, 1923*c*; Domm, 1927 and 1929; and others). Although the growth period of the right ovary in the combination graft is very short in comparison to the time required for the inversion following ovariectomy, it would be expected that at least the early phase of the process might be detectable histologically.

On the contrary, the left indifferent gonad primordium having a female-determining constitution exhibits considerable variation in its capacity to self-differentiate under similar developmental conditions. Twenty-one, or 53 per cent, of such left rudiments were capable of differentiating into left ovaries which closely resemble the normal left ovary of corresponding age. This capacity for self-differentiation was expressed even though the left gonad rudiment was associated with testicular tissue and grown on a male host. Nineteen, or 47 per cent, differentiated into left ovaries which show a marked reduction in size involving both medullary and cortical components, but especially the latter. In these cases the germinal epithelium becomes flattened, and cortical cords may be few or entirely lacking.

Statistical examination of the data points to a positive correlation between the occurrence of atypical left ovaries and their residence on male hosts. If such is the case, the sex of the host can be considered

as only one contributing factor in influencing the course of differentiation of the engrafted rudiment. This conclusion is based on several facts: (1) Left ovaries, quite normal in appearance, occur in grafts which also contain older testicular tissue and which develop on male hosts. (2) A modified left ovary may arise from a left gonad rudiment associated with left ovarian tissue and grown on a female host. (3) Left ovaries possessing a flattened epithelial covering and showing a marked deficiency in cortical elements arise from left gonad rudiments transplanted alone to female hosts.

Sixteen gonad rudiments (eleven left and five right) gave rise in combination grafts to gonad-like bodies whose sex cannot be definitely ascertained. Since the originally determined sex of the gonad rudiment is known in only five of these cases the data are inconclusive on the question of a possible inhibition of the rudiment's self-differentiating capacity due to hormones secreted by the associated gonad or by the host. The following evidence strongly indicates that these gonad-like bodies do not owe their origin to gonad rudiments developing under the influence of antagonistic sex hormones: (1) Indifferent gonad rudiments which have a male-determining constitution give rise to gonads of unspecific sex, although subject to hormones from associated testicular tissue and from a male host. (2) Similar gonad-like structures whose sex cannot be definitely ascertained arise from gonad rudiments transplanted alone and grown on either male or female hosts.

The originally determined sex of the left gonad which possesses structural characteristics of both a testis and an ovary (Plate II, Fig. 8) was not definitely ascertained, because of the loss of its control partner. It is highly probable, however, that the rudiment possessed a male-determining constitution and that the germinal epithelium persisted for several days after male sex cords had differentiated. Such a persistence of the germinal epithelium on the surface of the left testis of a chick embryo of 12 days' incubation was reported by Laulanié (1886), and the left testis of a late duck embryo described by Burwell (1931) exhibited a similar condition. Persistence of the germinal epithelium on the surface of the right testis has not been reported.

Willier, Gallagher, and Koch (1935) have experimentally stimulated the proliferation of cortex on the surface of the left testis of the chick by injecting theelin, theelol, or male hormone (urine derivative) into the albumin of the incubating egg. The right testis, on the contrary, does not respond to the hormones injected. These investigators attribute the difference in response between the two testes to the presence of a germinal epithelium which develops only on the left testis.

Since the left gonad shown in Plate II, Figure 8, possesses a germinal epithelium but no cortical cords, its origin can perhaps be more correctly attributed to the normal persistence of the germinal epithelium than to a response to hormones.

Although, based on statistical evidence, a positive correlation exists between the occurrence of atypical left ovaries and their residence on male hosts, we are faced with the fact that similar modified left ovaries arise in grafts which are grown on female hosts. It seems necessary to assume, therefore, that some other factor may strongly contribute to this disturbance of the normal course of differentiation which results in modified left ovaries and in the occurrence of gonad-like bodies whose sex cannot be ascertained. This factor may be purely a mechanical one.

Buyse (1935) finds that indifferent gonads of the rat embryo, when transplanted to a subcapsular position in the kidney of an adult male rat, give rise to: (1) testes essentially normal in appearance, (2) ovaries approximating normal structure, (3) ovaries which may show a marked deficiency in cortical elements, (4) ovotestes, and (5) gonads whose sex cannot be ascertained. That this modification is not a "free-martin" effect was proved when similarly modified ovaries were recovered which had arisen from undifferentiated gonad rudiments transplanted to female hosts. Buyse concludes that the modification is probably due to a high susceptibility of the cortical tissue to unfavorable mechanical factors in the graft.

The cortical reduction exhibited by the modified left female gonads in these experiments may result from unfavorable developmental mechanics operating in the graft to which the cortex is more susceptible than is the medullary tissue. In case the mechanical factors are sufficiently unfavorable, normal growth and differentiation

may be inhibited to such an extent that lack of structural organization makes identification of the sex of the gland impossible.

With the exception of a possible influence exerted on the differentiating left female rudiment by its male host, the lack of specific response to hormones on the part of the gonads in the various types of graft and host-graft combinations is striking. Gonad rudiments originally determined male and right rudiments originally determined female, when transplanted in juxtaposition with testes and ovaries from embryonic chicks and grown on either male or female hosts, evidently may differentiate according to their original plan of organization, irrespective of hormones to which they are subjected. Since 28.5 per cent of the left ovaries having a typical structure arose in combination grafts which developed on male hosts, a specific response of the left female rudiment to hormones secreted by the male host may even be questioned.

Ovarian and testicular tissue, known to be producing hormone at the time of transplantation, show no greater capacity for influencing the direction of differentiation of the gonad rudiment in the same graft than do embryonic ovaries and testes. The fact is thoroughly appreciated that no proof exists that the older gonad tissue continues to secrete hormone when transplanted to the membrane. Nevertheless, it is necessary to point out that, in case hormone secretion continues, the hormone is either produced in too small a quantity or does not have the capacity to influence the direction of sex differentiation in the associated gonad rudiment.

Ovaries and testes differentiated as to sex at the time of implantation develop in close proximity or in contact in heterosexual combination for a period of 8 or 9 days and continue to develop in essentially a normal manner.

These facts are interesting when viewed in the light of modifications which occur in the gonads of heterosexual pairs of certain parabiotic amphibia, owing, according to Witschi (1931 and 1934), to an antagonism between cortical and medullary elements which are in close proximity. The data would seem to justify the conclusion that in the chick no such direct local effect is exerted by one gonad upon another of opposite sex as the result of close association in space over a 9-day growth period.

The lack of response on the part of the reproductive organs of the host embryo to the various types of gonad grafts developing on the chorioallantoic membrane corroborates the findings of Willier (1925 and 1927), Kemp (1925 and 1927), Willier and Yuh (1928), and others. Even "multiple-testis" grafts interposed in the blood circulation of the host embryo failed to modify the structure of its reproductive glands and ducts. This does not necessarily imply that these organs are not capable of a response to large quantities of male hormone, but that the dosage necessary for such a response is greater than that which may be supplied by transplantation of the living testicular tissue to the membrane.

SUMMARY

1. Morphologically undifferentiated chick gonad rudiments and older ovarian or testicular tissue were transplanted together on the chorioallantoic membrane of 8- or 9-day embryos, to determine what influence the sex of the associated gonad or of that of the host might have upon the rudiment's capacity to self-differentiate. To ascertain the originally determined sex of the gonad rudiment in the combination graft, its partner gonad from the same donor embryo was transplanted alone to a second host.

2. Right and left rudiments originally determined male and right rudiments having a female-determining constitution were found to possess the capacity to self-differentiate into testes and right ovaries, respectively, regardless of the sex of the associated gonad and of that of the host.

3. Left rudiments, originally determined female, gave rise in such grafts to left ovaries, 53 per cent of which correspond closely to the normal. The remaining 47 per cent exhibit structural modifications involving principally the cortical elements. Although a modified left ovary may develop in combination with left ovarian tissue on a female host and with testicular tissue on a male host, statistical evidence points to a positive correlation between atypical left ovaries and their residence on a male host.

4. Five right and eleven left gonad rudiments in combination grafts gave rise to gonad-like bodies of unspecific sex. These were associated with either ovarian or testicular tissue and developed on

both male and female hosts. Similar structures arise from gonad rudiments transplanted alone to either male or female hosts.

5. Some factor other than the sex of the associated gonad or the sex of the host evidently contributes to the occurrence of the modified left ovaries and the gonad-like bodies whose sex cannot be ascertained. This factor is probably unfavorable developmental mechanics operating in the graft.

6. No evidence was found of a local effect of cortical and testicular (or medullary equivalent) elements upon each other. This is true for combinations of differentiated gonad tissue of opposite sex, as well as for differentiated and undifferentiated gonad combinations.

7. Gonad tissue known to be producing hormone at the time of transplantation is no more effective in altering the course of differentiation of the gonad rudiment in the same graft than is gonad tissue from embryos.

8. Although slight variations in structure occur in the reproductive organs of the host embryos, they are all within the range found characteristic of the normal embryo.

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CATALASE ACTIVITY DURING EMBRYONIC DEVELOPMENT (ACRIDIDAE, ORTHOPTERA)[1]

(Three figures)

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SEVERAL theories exist as to the possible functions of the enzyme catalase. The earliest of these considered its activity to be directly correlated with respiration and general metabolism (Alvarez and Starkweather, 1918; Appleman, 1916, 1918; Burge, 1922, 1926; Burge and Leichsenring, 1922; Davis, 1930; Gustafson, Clark, Shaw, and Warweg, 1932; Neller, 1931). Other investigators have found no indication of such a correlation (Alexeeff and Russinowa, 1931; Bialascewicz, 1921; Bodine, 1921; Crocker and Harrington, 1918; Doyle and Clinch, 1928; Morgulis, 1921; Pope, 1933; Rhine, 1924; Stehle, 1919; Stern, 1927). More recently Dixon (1925) has shown that the enzyme acts as an antitoxin by decomposing hydrogen peroxide produced in certain cellular oxidations. Callow (1923) marks a similar observation based on differences in catalase activity of aërobic and anaërobic organisms (Kluyver, 1924; Morinaga, 1925; Stern, 1927). Lantz (1927) thinks the function of the enzyme may be to prevent excessive oxidation, while Staffe (1931) suggests that it may have an oxygen-sparing action.

Several workers have investigated catalase and its relation to cellular activity during the course of embryonic development of insects (Fink, 1930; Spooner, 1927; Steche and Waentig, 1912; Zeiger, 1915). However, since little definite information is available about this subject, a detailed study of changes in catalase activity during the entire embryonic development of *Melanoplus differentialis* has been made. The egg of this grasshopper has proved to be particularly suitable for such an investigation. This egg, in its normal course of development, passes through a period of marked cellular activity, followed by a resting or diapause stage, after which

[1] Aided by a grant from the Rockefeller Foundation for work on cellular physiology.

active development continues up to the time of hatching. The diapause, or developmental block, may be broken experimentally by exposing the egg to a low temperature, such as 5° C., for an appropriate period of time. Since rates of oxygen intake have been established and correlated with the various developmental activities of this egg (Bodine, 1929; Bodell, 1935), it seemed desirable to investigate the development of, and the possible rôle played by, the egg catalase.

Catalase activity has been measured by the oxygen produced from the decomposition of hydrogen peroxide. The volume of this gas was determined by means of the especially adapted mercury manometer shown in Figure 1. The manometer vessel (*A*) is equipped with a side chamber (*B*), making it possible to keep extract and hydrogen peroxide separate within the vessel. Paired openings (*C* and *D*), one in the neck of the flask and one in the base of the manometer, may be superimposed, thus leaving the vessel open to the air during temperature equilibration. By rotating the vessel, the system is closed off from the outside.

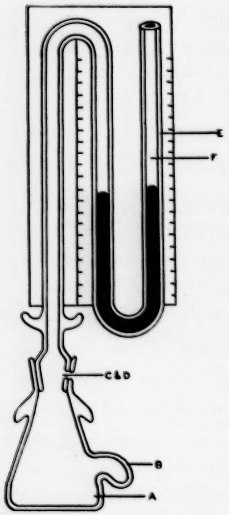

FIG. 1.—Type of manometer used in experiments. Volume of vessel (*A*), 25 cc.; volume of side chamber (*B*), 0.8 cc.; length of tube (*E*), 220 mm.; diameter of tube (*F*), 2 mm.

In all experiments, twelve manometers were placed on a mechanical shaker, in a water bath kept at 25° ±0.2° C. At the end of a 10-minute equilibration period, they were closed and set in motion, thus thoroughly mixing the contents of the flasks. In all cases the end-point of the reaction was reached within an hour, and this length of time was considered a minimum for the duration of experiments.

Eggs used were of three types: (1) those kept constantly at 25° C., on moist filter paper; (2) those exposed to low temperatures (5° C.) during the pre-diapause period and later placed at 25° C.; and (3) those exposed to 5° C. during diapause and subsequently kept at 25° C.

The average developmental stage of any series of pre-diapause eggs was determined by removing the embryos from a ten-egg sample. Morphological stages of the embryo in diapause and post-diapause eggs were determined by noting eye position or by removal of the chorion overlying the transparent cuticle (Slifer, 1932). Chorion removal has no effect on catalase activity, as eggs used with or without it gave similar results.

Since embryos in diapause and those in early post-diapause are morphologically similar, the physiological states of such eggs were determined by measuring respiratory rates in a differential manometer (Bodine, 1929).

Enzyme extracts were prepared by grinding one or more eggs with 2.5 cc. of distilled water in a glass mortar. Eggs ground with or without sand gave similar results. An 0.8-cc. portion of the extract was used in each determination, and catalase activity has been expressed as cubic centimeters of oxygen per egg.

Hydrogen peroxide solutions were made up daily and adjusted with phosphate buffer to a pH of 6.8–7.0, which for general purposes may be considered an optimum hydrogen-ion concentration range for the catalase reaction (Morgulis, 1921). The buffered peroxide was kept in colored glass bottles, and standardized daily with N/0.1 KM_nO_4. Figure 2 shows graphically the relation between the amount of oxygen evolved and the concentration of hydrogen peroxide when the same egg extract was allowed to react with varying hydrogen peroxide concentrations. From an inspection of this figure, it will be noted that the maximum gas evolution occurs when N/0.24–0.40 H_2O_2 is used.

In each experiment, a blank manometer containing only hydrogen peroxide and distilled water or boiled extract was prepared in order to correct results for possible changes in atmospheric pressure and spontaneous decomposition of peroxide. If sand had been used in the preparation of test extracts, it was also included in the control manometer.

Oxygen volume was calculated from manometric readings as follows: $(R_1 - R_0)K = V$, where V equals volume of oxygen in cubic centimeters; R_1 and R_0, total millimeter change in the readings of test and control manometers, respectively; and K, the constant of

the manometer for oxygen at standard conditions of temperature and pressure.

The amount of variation in total oxygen volume due to individual differences in manometers, and perhaps to irregularities of technique, has been repeatedly determined by using uniform extract and hydrogen peroxide in a series of eleven manometers. The maximum variation in gas volume in any one experiment did not exceed 0.0011 cc.

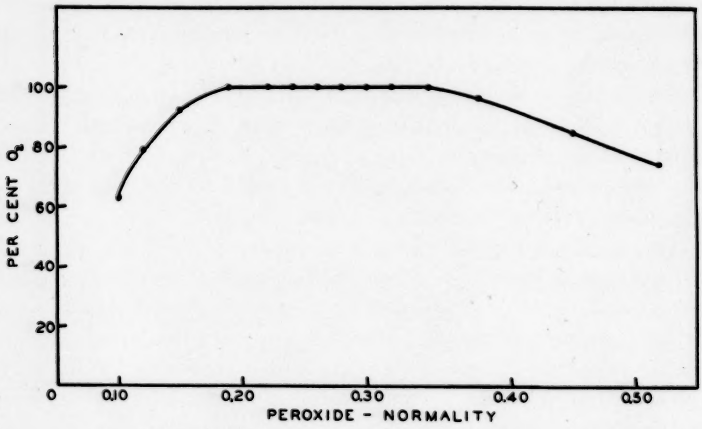

FIG. 2.—Catalase activity with variations in H_2O_2 concentration (100 per cent equals optimum oxygen production).

RESULTS

The relative catalase activity and respiration of the eggs is shown graphically in Figure 3. Each point indicated represents the average value for a group of at least fifty eggs. It will be noted that from the time of laying, through the eighth day of development, there is practically no change in catalase activity. During this period, ten eggs were used in the preparation of each extract in order to obtain significant readings. In all subsequent stages, however, individual eggs were employed. From the ninth to the twenty-first day, or through pre-diapause, a marked increase in catalase activity occurs. During early diapause (23–30 days) it is unchanged, but from the thirtieth to the fortieth day it increases rapidly. Statistical compari-

son of data covering the 28–32 and the 39–41 day stages indicates significant differences in catalase activity between these two periods. Maximum catalase activity reached on the forty-first day continues throughout diapause. All post-diapause determinations have been made on eggs which were kept at 25° C. after they had been exposed

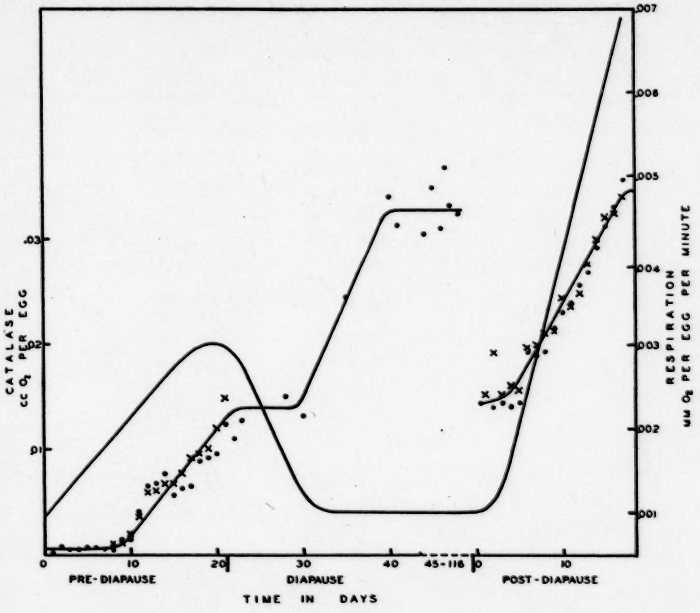

FIG. 3.—Catalase activity and respiratory rate of eggs from laying to hatching. Ordinates: at left, cubic centimeters of O_2 evolved from H_2O_2 by catalase per egg; at right, relative O_2 intake per egg per minute. Abscissa, days of development. Dots, eggs exposed to 5° C. during diapause. Crosses, eggs exposed to 5° C. during pre-diapause. Respiratory curve (without dots or crosses) adapted from Bodine (1929).

to cold (5° C.) for several months in order to remove the diapause block. Catalase activity during the first 5 days of post-diapause remains at the level which is characteristic of early diapaues (23–30 days). From the sixth day of post-diapause until hatching, the amount of catalase in the egg increases rapidly to the maximum. There was no change shown in catalase activity at the time of hatching or during the first instar.

It will be noted that throughout developmental periods there is no difference shown between the catalase activity of eggs which had been exposed to cold during pre-diapause and that of eggs not exposed to cold until in the diapause stage.

An attempt has been made to determine the relative distribution of catalase within the egg. Separation of yolk from embryo is easily accomplished in all stages of development. After yolk engulfment, the yolk may be freed from the embryo by puncturing the posterior

TABLE I

DISTRIBUTION OF CATALASE ACTIVITY BETWEEN (1) EMBRYO AND (2) YOLK PLUS EXTRA-EMBRYONIC EGG MEMBRANES AND FLUIDS

(Each figure represents average result from twenty eggs)

Embryonic State	Percentage of Catalase Activity	
	Embryo	Yolk Extra-embryonic Membranes and Fluids
10 days	5	95
15 days	5	95
Diapause (50 days)	5	95
Post-diapause (15 days)	65	35

end of the abdominal cavity and forcing the yolk out through the aperture. As is indicated in Table I, during pre-diapause and diapause the yolk, plus egg membranes and fluids, contains 95 per cent of the total catalase activity, only 5 per cent being found in the embryo. However, after yolk engulfment, or in late post-diapause, the embryo (measured 2 days before hatching) contains 65 per cent of the enzyme, and the yolk 35 per cent. Only in rare cases was catalase activity observed in extracted shells. It was assumed that such activity in shells was due to incomplete removal of the yolk and fluids. It seems, therefore, that most of the catalase is concentrated in the various extra-embryonic fluids and cellular structures until after yolk engulfment, when the situation is reversed.

If one compares relative catalase activity with changes in the respiratory rates of similar eggs (Bodine, 1929; Boell, 1935), it is evident, as shown in Figure 3, that no correlations seem to exist.

As no mitotic spindles are found during diapause (Slifer, 1931) and as catalase activity is high during this period, there seems to be no apparent or significant correlation between cell activity and catalase.

SUMMARY

1. A quantitative study has been made of catalase activity in the egg of the grasshopper, *Melanoplus differentialis*, throughout its entire period of development.

2. Catalase activity is at a minimum for 8 days after laying. It increases from the ninth to the twenty-first day, is fairly constant from the twenty-first to the thirtieth day, and rises (30–40 days) to reach a maximum (40 days) retained throughout the remainder of diapause. After diapause, in eggs which have been cold-treated, catalase is at the 21–30-day level for a period of 5 days. From the sixth day of post-diapause until hatching, the enzyme increases to a maximum.

3. Exposure to low temperature (5° C.) has no effect on the subsequent catalase activity (at 25° C.) of pre-diapause eggs.

4. Before yolk engulfment, 95 per cent of catalase activity is found in the yolk, egg membranes, and fluids, and 5 per cent in the embryo; after engulfment, 65 per cent in the embryo, and 35 per cent in the yolk, extra-embryonic structures, and fluids.

5. No correlation seems to exist between respiratory rate and catalase concentration or activity.

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RECIPROCAL INHIBITION AND ITS REVERSAL BY STRYCHNINE IN THE MODIFIED CTENOPHORE, COELOPLANA BOCKII[1]

(Two figures)

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OF FIRST importance in maintaining the integrity of muscular movement in the individual are two properties of the nervous system, namely, rapid conduction of impulses and reciprocal inhibition. The first is apparent wherever nervous tissue is found; the second occurs where muscles are paired so that if both contract at the same time they act antagonistically and nullify movement. Reciprocal inhibition apparently is absent in coelenterates but occurs in echinoderms and worms. It therefore becomes a nice question to determine to what degree anatomical complexity of the nervous system is necessary for this function. In the vertebrate, where the problem has been most studied, the arrangements for reciprocal inhibition in voluntary movements are exclusively central. On the other hand, the muscles which open and close the pincers of the great claw of the crayfish show reciprocal inhibition even when the appendage is detached; hence the mechanism in this case is peripheral. It is also of interest to note that the intestine of the vertebrate, in which the musculature is innervated by a nerve-net plexus, shows reciprocal inhibition in the involuntary movements of peristalsis. Therefore, the plexus must be adequate to the reaction in this highly special case. In fact, Magnus and Wolf (1913), as a result of their work on the reflexes of the skeletal musculature of the mammal, concluded that reciprocal inhibition cannot be explained on the basis of any certain anatomical scheme of nervous

[1] It gives me pleasure to express my heartiest thanks to Mr. M. Eri and Mr. Y. Yoshii for many courtesies extended to me during my stay at Misaki, and to Professor Komai for helpful advice on the structure of *Coeloplana*.

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connections. Reciprocal inhibition, therefore, must depend upon a special functioning of a sensory-motor neuron pattern, which may vary in its form in representatives of the different phyla.

Echinoderms, worms, mollusks, and crustaceans all show reciprocal inhibition. In coelenterates the nerve-net system apparently functions exclusively in conducting and shows inhibition only when excitation is applied so that two impulses arrive at an effector from opposite directions (Moore, 1926). The evidence goes to show that ctenophores behave in the same way. I found it, therefore, of considerable interest to study the reactions of a creeping form of the modified ctenophore, *Coeloplana bockii* (Moore, 1933), with a view to discovering whether, in a form structurally so elementary, reciprocal inhibition plays a part in locomotion.

The nervous system of *Coeloplana* is, according to Komai (1922), of the simplest net type, without any evidence of concentration into ganglia or strands. Yet, reciprocal inhibition can be demonstrated in this form. The adult *Coeloplana* is a flat, slowly creeping animal which superficially resembles a flatworm. It is found living symbiotically on colonies of *Alcyonaria* in the shore waters of Indo-China and southern Japan. The free-swimming larva is, in appearance, a typical ctenophore with the characteristic eight rows of paddle plates. The body of the adult is formed from the embryo by the outward growth of the pharynx, a process which begins as soon as the young animal settles down with its oral opening in contact with a surface. As this development proceeds, the paddle plates disappear and only the two branched tentacles remain to bear witness to the animal's ctenophore history. The tentacles determine the long axis and the bilateral symmetry of the animal; but in creeping, it may move equally well in any direction—sidewise, forward, or backward.

The experiments to be described were carried out on a large number of individuals in June and again in November at the Misaki Station. The results were uniformly the same in all cases, and the facts described are therefore well established.

If an active *Coeloplana* is touched at any point on its surface—say the lateral margin—that region moves away by withdrawing centralward as a result of the contraction of the transverse musculature, while the opposite side extends by virtue of the relaxation of its

transverse musculature, the total effect of both movements being to carry the animal away from the point of excitation (Fig. 1). This constitutes a simple case of reciprocal inhibition as regards the transverse musculature of the contralateral side. The pattern of the response is changed, however, if the animal is kept for a few minutes in a solution of strychnine sulfate in sea water, 1 : 50,000.[3] If it is now stimulated on the lateral margin, as in the first case, there is the usual

FIG. 1

FIG. 2

FIG. 1.—Normal specimen of *Coeloplana* responding to mechanical stimulation applied at the locus indicated by the arrow. The result: withdrawal toward center by the ipsilateral side and movement away from the center by the contralateral side.

FIG. 2. Strychninized specimen of *Coeloplana* responding to mechanical stimulation as in Figure 1. The result: withdrawal toward center by both sides, showing reversal of response under strychnine for the contralateral side.

withdrawal from the locus of excitation toward the center; but the opposite side also reacts in an identical fashion by moving centralward, which actually carries that side *toward* the locus of excitation (Fig. 2). This is the result of the contraction of the contralateral transverse muscles which, in the normal response, are inhibited. Strychninization has thus changed the reciprocal inhibition of the muscles of the contralateral side into an excitation—a conversion which causes *Coeloplana* to react to stimulation from a single point just as a medusa does, by a bending of the body toward that spot.

[3] Atropine sulfate, caffeine, nicotine, and phenol in dilute solutions in sea water cause hyperexcitability and spasmodic contractions of the musculature in *Coeloplana*, effects similar to those produced by strychnine. Only atropine, however, yields reversal effects comparable to those of strychnine.

Specimens of *Coeloplana* in which a central disk, including the "aboral sense organ," had been excised, reacted in every way like an intact animal. From this it is concluded that the nerve net of the body is adequate to mediate reciprocal inhibition and strychnine reversal. *Coeloplana* thus furnishes an instance of a form, without morphological evidence of central tracts or ganglia, which possesses neurons which are functionally and chemically developed so that they react in a typical way to bring about reciprocal inhibition and its reversal under strychnine. This is, however, not a common property of sensory and motor neurons, for sea anemones have both; but I have never been able to observe any excitatory effects of strychnine on the body musculature of these animals, even when they were kept for a time in a concentrated solution of strychnine in sea water.

The experiments with strychnine are interesting, as they throw some light on the question of the extent to which strychnine reversal of reciprocal inhibition takes place. In recent years a number of exceptions have been found to the original rule of Sherrington (1898), according to which the inhibition phase of reciprocal inhibition is transformed into an excitation by strychnine. Dusser de Barenne (1933) lists the exceptions to the rule. Thus, the tonic neck-reflexes, the compensatory eye-reflexes, and the caloric, labyrinthine eye-reflexes are unaffected by strychnine even in doses large enough to produce convulsions. Although strychnine reversal does not occur in these reflexes, the same muscles show typical reversal when activated in other reflexes. Similarly, inhibition of the extensor muscles in decerebrate rigidity, which is elicited by stimulation of the anterior parts of the cerebellar cortex, and inhibition of the opening muscles of the mouth in the mandibular and linguo-mandibular reflex are not reversed by strychnine. For these reasons Dusser de Barenne suggests that reciprocal inhibition which can be reversed by strychnine represents a special case in which "the inhibitory stimulus applied to the afferent peripheral nerve includes also an excitatory factor, which normally does not predominate, but under strychnine becomes dominant and blocks the inhibitory effect, whereas the absence of this reversal is probably due to the pure inhibitory nature of the excitation." It should be borne in mind that the exceptions to strychnine reversal occur in the vertebrates, chiefly mammals, and, so far as we

know, are confined to that phylum. The fact that reciprocal inhibition and its reversal by strychnine are of such general occurrence among the invertebrates, it seems reasonable to think, puts the matter in a somewhat different light. Since reciprocal inhibition can be shown to occur in *Coeloplana*, starfishes, flatworms (Moore, 1918), and annelids (Knowlton and Moore, 1917), we have sufficient evidence of its widespread distribution in the most diverse morphological types of nervous system. That strychnine reversal is the rule in all these cases indicates the fundamental nature of the reaction. It is suggestive and possibly significant that in the mammal the "recruitment" reflexes, which resemble those of the invertebrates in their long latency, slow onset, long after-discharge and slow decline, are profoundly affected by strychnine (Bremer and Rylant, 1926). It would seem, therefore, that the mechanism for pure inhibition which is not susceptible of strychnine reversal is a later, special development in the vertebrate.

The results with *Coeloplana* furnish an instance of extreme simplicity of neuronic structure which is, nevertheless, adequate to function as typical reciprocal innervation in locomotor movements. This gives support to the position of Magnus and Wolf according to which reciprocal inhibition depends for its mediation upon physiological, rather than special anatomical means. It may be regarded as established that for the mediation of reciprocal inhibition, sensory and motor neurons linked together in very simple histological pattern are adequate, and that in their chemical affinities, as indicated by the results of strychninization, they are equal to neurons of similar function in more complicated nervous systems.

SUMMARY

1. Specimens of *Coeloplana bockii*, which possess only a nerve-net system, show reciprocal inhibition of the transverse musculature as a result of contralateral excitation.

2. In strychninized specimens reciprocal inhibition is reversed and excitation results from contralateral stimulation.

3. Individuals from which a central disk, including the "aboral sense organ" had been removed, reacted similarly to the intact animals. Hence the neural reactions are mediated by the diffuse system of the body.

4. The results of the experiments support the view of Magnus and Wolf to the effect that reciprocal inhibition depends for its mediation upon physiological means rather than upon special anatomical arrangements.

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KILLING ORGANISMS WITH CHROMIUM AS FROM INCOMPLETELY WASHED BICHROMATE–SULFURIC-ACID CLEANED GLASSWARE

(One figure)

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POTASSIUM BICHROMATE is an important component of many of the killing and fixing fluids of the biologist, as well as of the bichromate–sulfuric-acid mixture often used for cleaning glassware. Because the bichromate adsorbs to glassware and is washed out with difficulty, it is necessary to investigate the danger involved when this mixture is used for cleaning glassware and other articles to be used with living organisms. Now that the amount of chromium which may remain after a certain amount of washing is known, the effect of this amount of chromium on certain test organisms is here reported.

I

Laug (1934) has found that S-diphenylcarbohydrazide may be used to determine colorimetrically as little as 0.1 μg.[1] of potassium bichromate per milliliter of solution. He placed sulfuric-dichromate cleaning fluid in different kinds of glassware for varying lengths of time. The dishes were then washed in seven changes of tap water and rinsed with three different lots of distilled water. After this washing, Laug proceeded to extract much of the remaining bichromate with different amounts of water remaining in contact for different lengths of time, and was able to recover, on the average, 0.025 μg. of bichromate per milliliter of wash water. When smaller amounts of water were used in the dishes, as in an experiment with living organisms, the bichromate concentration might become as high as 1.0 μg./ml. even after ten washings of the glassware. This makes it necessary to determine whether amounts of from 0.1 to 1 μg. of bichromate per milliliter of solution are toxic to living organisms.

[1] A microgram (μg.) or gamma (γ) is 10^{-6} gram.

II

The first test organism used was the yeast *Saccharomyces cerevisiae* Hansen because of its convenience and the standard conditions known for yeast growth (Richards, 1932, 1934). In each series part of the tubes of Williams' culture medium were retained as controls, and to the other tubes various known amounts of potassium bichromate were added.[2] The results from three separate series of experiments are summarized in Figure 1. The ordinate values give the percentage of yeast in the tubes of a given bichromate concentration of that of the control tubes as measured with a photoelectric nephelometer (Richards and Jahn, 1933). With very dilute solutions of potassium bichromate (0.0025 to 0.04 μg./ml.) the bichromate adsorbs to the glass and gradually redissolves into the culture fluid, and the bichromate shows greatest toxicity about 60 hours after the populations were seeded. As the cells are killed, the bichromate seems to be bound by the killed cells; and the resulting decreased concentration of the bichromate permits nearly complete recovery of the population of yeast. With stronger concentrations the amount of killing is greater at first; and as the bichromate is removed by the killed yeast, there is a corresponding recovery which is less complete as the concentration of the bichromate increases. A concentration of about 10 μg./ml. of bichromate sterilizes the culture.

It was shown (I) that the amounts of bichromate that might be found after ten washings of the glassware were from 0.1 to 1 μg/ml. These concentrations reduce the yeast populations from 22 to 47 per cent within 24 hours after seeding, and the ultimate recovery is not complete. The reliability of yeast measurement is such that deviations of 4 or more per cent indicate real differences beyond any normal variation. Consequently, concentrations as low as 0.0025 μg./ml. would invalidate experiments made with yeast.

III

Spirogyra insignis Hess and *S. tenuissima* Hess placed in dilute Knop's solution appeared normal and grew well for the 15 days' duration of the experiment (Table I). When the test amounts of 0.1 to 1 μg./ml. bichromate are added to similar amounts of Knop's

[2] For the effects of massive doses of $Cr_2(SO_4)_3$ on yeast, cf. Hébert (1907).

solution, one-quarter of the filaments placed in the test solutions are injured or killed within a day. These injured filaments bind and

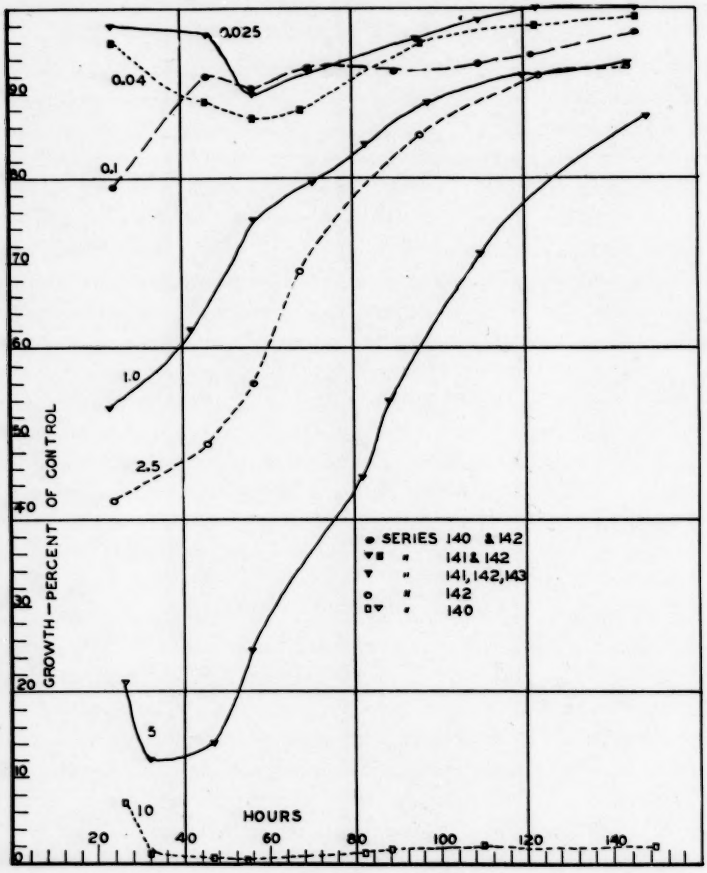

FIG. 1

reduce the poison; and later, with the lesser concentration, the plants nearly recover to the same condition as in the controls; but with the greater concentration only half of the algae recover. Fewer filaments of the smaller species recover than of the larger

species. In neither case was more than fair preservation accomplished by 100 μg./ml. While greater concentrations were not tested, it would appear that less bichromate than usually found in killing

TABLE I

	Control	0.1 μg.	1 μg.	5 μg.	10 μg.	50 μg.	100 μg.
Spirogyra insignis: 23 hours..	Normal and growing	25 per cent killed and blackened	25 per cent killed	Dead and slightly plasmolyzed	Plasmolyzed	Plasmolyzed	Killed and fixed
48 hours..	Same	Same	35 per cent killed	50 per cent killed	Same	Same	Same
120 hours.	Same	15 per cent dead	50 per cent killed	95 per cent killed	Plasmolyzed	Same	Fair preservation
15 days...	Same	90 per cent recovered	50 per cent normal	All dead	10 per cent preserved	20 per cent preserved	Fair preservation
Spirogyra tenuissima	Normal and growing	This species is more sensitive to the poison and is less well preserved in the higher concentrations. Only half of the filaments recovered in 0.1 μg./ml. in 15 days					
Oscillatoria limosa: 23 hours..	Actively motile	Motile	Motile	Motility reduced	No motility	Killed	Killed and fixed
120 hours.	Actively motile and growing	Few moving cells	80 per cent dead and plasmolyzing	All dead and plasmolyzing	All dead and plasmolyzing	Few preserved	70 per cent preserved
15 days...	Same	50 per cent motile	25 per cent motile	5 per cent motile	All disintegrated	Fair preservation	
Raphidium sp.: 120 hours.	Active	Less active	Few motile	4 per cent dead	50 per cent dead and disintegrating	99 per cent dead; fair preservation	
15 days...	0 per cent dead; active motility	12 per cent dead	5 per cent dead	25 per cent dead	67 per cent dead	80 per cent dead	90 per cent dead
		Motility greatly reduced with those still living				Fair preservation of dead	
Chlorococcus sp.....	Up to 5 μg./ml. little effect was noticed. Greater concentrations kill and none survived the higher concentration; and preservation was poor						

and fixing solutions might be used to advantage for the preservation of algae.

The blue-green alga *Oscillatoria limosa* Agardhi was tested in the same manner as *Spirogyra;* and while the injury was somewhat less during the first day, the later injury to the filaments was greater and

less recovery took place than with *Spirogyra* at the end of 15 days (Table I). Both of these algae are severely injured in amounts of bichromate that might be found when only ten washings were used following the use of sulfuric-bichromate cleaning fluid.

The diatoms and desmids tested (Table I) are less injured by such small amounts of potassium bichromate than the blue-green and green algae. With the diatom (*Raphidium*), the upper possible limit (1 μg./ml.) may produce sufficient injury or killing to vitiate the use of it as a test organism in incompletely washed bichromate–sulfuric-acid-cleaned glassware.

IV

The danger of bichromate poisoning from inadequately cleaned glassware is less when the culture fluids are alkaline, because less bichromate will remain in solution; and this is true also for sea water. Ten micrograms per milliliter and greater concentrations in sea water were found to be toxic for the developing sea-urchin egg (*Arbacea punctulata* Lamarck).[3] Until the toxicity of bichromate to other marine organisms is known, care should be taken to avoid the possibility of bichromate contamination from inadequate washing of vessels treated with bichromate–sulfuric-acid solution.

V

The effect of potassium bichromate on the developing *Amblystoma punctatum* egg was determined by placing the eggs in tap water containing known amounts of the chemical and comparing the development with controls in the tap water without the chemical. A very small amount (0.0001 μg./ml.) delayed the development at first by about two Harrison stages, and a few days later the animals appeared to be four stages less developed than the control animals. The larvae hatched a day or two earlier than the control animals because the capsule was weakened by the bichromate. About half of the larvae developed a kyphotic bending of the body shortly after hatching; and their swimming movements were irregular, leading to circus movements, or to tetanic paralysis on slight stimulation. The total mortality was about 16 per cent, while none of the control animals were dead when the experiments were terminated within a

[3] I am greatly indebted to Dr. Ethel Brown Harvey for making the tests with *Arbacea*.

week after hatching. About half of the animals appeared to be uninjured, but most of these were one stage less developed when they hatched.

With concentrations of 0.001 μg./ml. and stronger, no normal animals appeared. This concentration retarded development about two stages, and the mortality was 33 per cent. The first evidence of injury appeared within 2 days as an incomplete closure of the neural folds and an extrusion of some yolk material. The injury led to marked deformities during development and to disturbed equilibrium and swimming of the tadpoles on hatching.

The same kind of early injury and deformity occurred when the concentration was 0.01 μg./ml.; the mortality was 36 per cent; and at the end of the experiment the average length of the larvae was 8 per cent less than that of the controls. A slightly higher concentration, 0.1 μg./ml., also resulted in early injury and deformity; and many of the animals were dead within a period of 4–10 days. The length of the gills was decreased by about 20 per cent; the total length was 10 per cent less; and the mortality was 55 per cent.

Increasing the concentration to 0.5 μg./ml. increased the total mortality to 80 per cent; and there was less early injury but greater later injury, which was probably due to a tanning of the mucin capsule retarding the penetration of the bichromate. Considerable reduction of the gills and a marked increase of mucus secretion on the surface of the animal were observed.

One microgram per milliliter, the greatest concentration that might occur when only ten rinsings of glassware were used, showed a definite hardening of the capsule and of the vitelline membrane. In one case the animal reached stage 40 and died without being able to rupture or dissolve the vitelline membrane. In most cases the animal was deformed in the capsule before hatching. Many were kyphotic; and others had frilled, deformed tails. If the animal appeared normal in form at hatching, the swimming movements were impaired and a twisting of the body occurred within 2 days after hatching. All of the animals had dwarfed and deformed gills and a hypersecretion of mucus. The average length of the animals at the end of the experiment was 12 per cent less than that of the controls, as nearly as one could measure around the deformed bodies to esti-

mate their length. The mortality was a little less, 60 per cent, because the tanning of the capsule lowered the early mortality; but none of the survivors were in any way normal.

Greater concentrations of potassium bichromate than 1 μg./ml. penetrated the hardened capsule still less, so that the early injury was less and the retardation of development averaged only one Harrison stage. Hatching was delayed from 1 to 5 days because of the hardened capsule, and the injury immediately following hatching quickly paralyzed the animals.

The developing salamander eggs are more sensitive to small amounts of bichromate than any of the other organisms used in the experiments. Sulfuric-bichromate cleaning fluid should never be used on glassware intended for the rearing of these animals.

VI

Can the adsorbed traces of bichromate which may be toxic to living organisms be removed after clearning with the sulfuric-bichromate cleaning fluid? Laug (1934) has demonstrated that the amount recovered in successive portions of wash water is greatest at first and gradually becomes less. Hot water was found to be over three times more efficient than cold water. He recommends successive periods of at least 15 minutes in boiling water as the best method for removal of the bichromate.

Small, nearly closed vessels, such as are commonly used in microrespiration apparatus (Warburg, etc.), are freed from adsorbed cleaning fluid with difficulty; and especial vigilance should be maintained to make certain that the washing is adequate. Improper washing of the vessels may be responsible for some of the erratic and divergent results sometimes obtained with this type of equipment.

Unless it is known that the washing is sufficiently complete or that the organisms used are not affected by the amount of bichromate remaining, it would seem better to avoid the use of this cleaning mixture. Ten per cent nitric acid will remove many of the residues found on glassware, washes out easily, and is volatile in case traces are not washed out. Another good cleaning solution is 1–5 per cent trisodium phosphate. This probably is not very toxic to organisms in amounts that would not wash out readily. The efficacy of many soap compounds is due to the addition of this alkali. The

writer has found that Sapolio, with the vigorous use of a good brush, is quite adequate for cleaning glassware to be used for living organisms, and that it washes off easily. Pipettes, etc., which cannot be reached by a brush may be cleaned by the less toxic compounds.

SUMMARY

Concentrations of from 0.1 to 1.0 μg./ml. of bichromate may occur when small amounts of water are used in dishes which have been cleaned with potassium-bichromate–sulfuric-acid cleaning fluid and then washed with seven changes of tap water and three of distilled water. Laug has further demonstrated the difficulty of removing these remaining traces of bichromate from glassware. The foregoing amounts of potassium bichromate are shown in this paper to be sufficiently toxic to invalidate experiments made with yeast, *Oscillatoria*, two species of *Spirogyra*, and developing *Amblystoma* eggs. A desmid and a diatom were less sensitive to potassium bichromate, but the former (*Raphidium*) was injured by 1.0 μg./ml. of bichromate. Only 50 per cent of the *Amblystoma* hatched as normal larvae when the bichromate concentration was 0.0001 μg./ml. Unless it is known that the washing of glassware cleaned in sulfuric-acid–bichromate fluid is adequate, or that the organisms used are not poisoned by such traces as may remain, it is recommended that less-toxic cleaning fluids be used. The cleaning of microrespiration apparatus should receive special attention. Other cleaning materials that wash off more easily are Sapolio, 10 per cent nitric acid, and 1–5 per cent trisodium phosphate.

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STUDIES ON THE PHYSIOLOGY OF AMOEBA I. THE RELATION BETWEEN NUTRIENT SOLUTION, ZONE OF GROWTH, AND DENSITY OF POPULATION

(Three figures)

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THE general culture conditions in a culture of amoebae, free of bacteria, were discussed in a previous study (Reich, 1935). It was observed there that in a semiliquid nutrient solution the amoebae grow in a distinctly formed zone of growth at a depth of 5–7 mm. below the surface. The purpose of the present study was to find out whether the adjustment to a relatively low oxygen pressure is a constant quality and could, as such, characterize the species, or whether it depends upon various external factors. The first experiments with different H-ion concentrations actually showed that the depth of the zone of growth may be affected. It could be seen, at the same time, that, as the density of population increases, the zone of growth descends farther and farther down. The question arose as to whether the depth of the zone of growth is indicative of the oxygen requirement of the amoebae or whether it depends simply on the weight of the amoeba layer, which becomes the heavier and sinks the deeper, the richer it is in individuals. It was necessary, therefore, to look for factors which would affect the number of individuals without changing thereby the depth of the zone of growth.

EXPERIMENTS WITH VARIOUS H-ION CONCENTRATIONS

In these experiments the nutrient solution previously indicated (Reich, 1935) was used with addition of 0.15 per cent agar. By adding small amounts of NaOH or HCl, the desired pH was obtained. Five cubic centimeters of this solution were poured into each of several test tubes, the diameters of which were equal as far as possible; and then the portions were sterilized. Special care was exercised to use only simultaneously prepared samples of nutrient

solutions in each series, in order to avoid errors which might result from minute differences in the composition of the solutions. The amoebae to be inoculated were taken only from well-growing cultures previously centrifuged and washed five times with 0.1 per cent NaCl solution. Each test tube was inoculated with 0.5 cc. of the amoeba suspension. The first experiment comprised the following H-ion concentrations: pH 8.2, 7.6, 7.0, 6.7, 6.6, 6.2, 6.0, 5.8, 5.7. Figure 1 and Table I illustrate the results of a one-month culture. The figure shows clearly that in an alkaline medium there exist differences in the depths of the zones of growth; whereas in solutions

FIG. 1.—The different depths of the zones of growth in cultures of amoebae of various H-ion concentrations. The numbers indicate the various pH values. The not-inoculated control medium of pH 6.8 is marked with *O*.

of pH 6.7 and less, the zones appear roughly at the same depth. A second characteristic feature to be mentioned is the fact that, while at pH 8.2 and pH 7.6 the downward distribution of the amoebae in the solution is uniform and an indistinct boundary appears only toward the top, from pH 7.0 on, the zone of growth is markedly limited, both below and above. The reason for the impression of a more pronounced upward boundary is probably that the downward-wandering amoebae drag the agar particles along with them, thereby effecting a clarification of the upper strata of the semiliquid medium. The test tube marked with an *O* in the figure serves as a control containing a not-inoculated nutrient solution of pH 6.8. It shows that this process of stratification is not a pure precipitation phenomenon of the agar colloidal particles but that it is brought about by the growth of the amoebae.

In order to determine the number of amoebae present, the cultures were well shaken and 10 drops of each culture were counted, drop by drop, in a Thoma counting-chamber. From each group two test tubes were selected, so that the numbers in the table represent the average values for every twenty counts and refer to 0.1 cu. mm. of solution.

In Table I are indicated the number of amoebae, the number of cysts, and the sum of the two. The table shows that the more acidic the solution the greater is the number of amoebae.

Two days after counting the amoebae, the normal stratification of the cultures reappeared. One month later, i.e., after two months

TABLE I

THE EFFECT OF H-ION CONCENTRATION ON THE DENSITY OF POPULATION IN A ONE-MONTH CULTURE

	pH								
	8.2	7.6	7.0	6.7	6.6	6.2	6.0	5.8	5.7
Amoebae	1.2	5.1	8.4	9.8	10.9	11.9	12.5	11.2	11.8
Cysts	0.2	0.6	1.4	3.0	2.2	4.7	3.3	3.8	4.2
Total	1.4	5.7	9.8	12.8	13.1	16.6	15.8	15.0	16.0

of culivation, the amoebae were again counted, the results of which are given in Table II. This table shows the differences in the densities of population still more clearly, for the number of amoebae changes in the alkaline solution only imperceptibly, whereas in the acidic solution it increases considerably. However, it proves that the time of counting the amoebae plays no important rôle in the experiment.

These experiments, as well as the previous ones, show that the appearance of the cysts depends in the first place on the age of the cultures. As the maximal density of population is constant, we can restrict ourselves to the sum totals in our further discussion without considering the relation between amoebae and cysts.

The results gave room for the possibility that by making the nutrient solution alkaline a substance necessary for growth and respiration precipitated out. In order to check this assumption, an

experiment with two parallel series was performed. The nutrient solution was divided into two equal parts. One part was made strongly alkaline, boiled, and filtered, and by adding HCl the desired pH was obtained. NaOH was added to the other strongly acidic part, so that this solution, also, acquired the desired pH. If the substance which precipitated should really be necessary for the growth and respiration of the amoebae, then, by filtering it, distinct differences between the two series should be apparent. However, not only did such differences not appear but, on the contrary, an agreement between the two series, comprising the six pH ranges from 7.6 to 5.5,

TABLE II

THE EFFECT OF H-ION CONCENTRATIONS ON THE DENSITY OF POPULATION IN A TWO-MONTH CULTURE

	pH								
	8.2	7.6	7.0	6.7	6.6	6.2	6.0	5.8	5.7
Amoebae.....	2.1	4.8	6.7	12.2	8.6	19.0	24.9	26.9	24.3
Cysts.....	0.5	2.7	6.2	13.8	18.6	19.8	15.5	16.5	19.6
Total.....	2.6	7.5	12.9	26.0	27.2	38.8	40.4	43.4	43.9

was observed. This proved that the H-ion concentration alone is the determining factor involved.

The purpose of the third experiment was to determine the limit of the tolerated H-ion concentrations. A nutrient solution of pH 4.8 was tolerated, although the growth-rate of the amoebae slackened perceptibly and the maximum attained was lower than that at pH 6.4. The depth of the zone of growth was apparently the same as that in other acidic cultures. An accurate determination could not be carried out, since the agar particles flaked out to a certain extent in not-inoculated cultures as well. In solutions of higher H-ion concentrations, the amoebae encysted. The attempt to make them grow under anaërobic conditions did not succeed.

EXPERIMENTS WITH VARIOUS SALT CONCENTRATIONS

A second series of experiments was arranged with nutrient solutions of different salt concentrations. At first the NaCl content was

varied in an otherwise unchanged nutrient solution of pH 6.8. The nutrient solution free of NaCl was boiled, filtered, and divided into two halves. One per cent NaCl was added to one half. The two halves were then mixed in different proportions, so that a series of concentrations of 10, 8, 6, 4, 2, and 0 NaCl per mille resulted. Table III and Figure 2 show the results. They prove conclusively

TABLE III

THE EFFECT OF DIFFERENT NaCl CONCENTRATIONS ON THE DENSITY OF POPULATION OF AMOEBAE

	NaCl					
	1.0 per cent	0.8 per cent	0.6 per cent	0.4 per cent	0.2 per cent	0.0 per cent
Amoebae	0.1	0.5	0.2	1.7	1.7	0.3
Cysts	1.8	1.7	1.6	12.4	15.0	14.7
Total	1.9	2.2	1.8	14.1	16.7	15.0

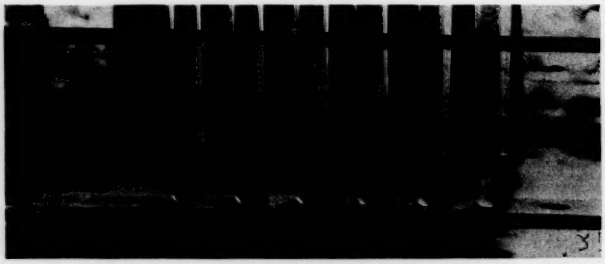

FIG. 2.—The different depths of the zones of growth in various salt concentrations. The numbers indicate the NaCl concentrations per mille.

that an increase in NaCl concentration has an effect similar to that of an increase in alkalinity, i.e., it causes a dispersed growth, a heightening of the zone of growth, and lowers, at the same time, the maximal density of population.

In other experiments the concentration of all salts was varied in an analogous manner, whereby a tenfold basic solution was the highest concentration used. These experiments yielded similar re-

sults. Finally an experiment was done by replacing the usual solution by artificial sea water of the following composition: 3.0 NaCl, 0.07 KCl, 0.26 $MgSO_4$, 0.50 MgCl, per cent, respectively. One per cent, respectively, peptone and glucose were added to this salt solution. The final solution was diluted with a 1 per cent solution of peptone and dextrose in distilled water; and the following concentrations of NaCl were tried out: 3, 2.4, 1.8, 1.2, 0.6, and 0.3 per cent, respectively, the other salts being diluted in the same proportions. This experiment corroborated the previously obtained results. A rich growth and a distinct formation of a zone of growth were observed only at 0.3 per cent NaCl concentration. The phenomenon is the more curious, as it was shown in a previous paper (Reich, 1933) that *Mayorella palestinensis* showed a rich growth in a nutrient solution containing bacteria, even at 3 per cent NaCl.

THE EFFECT OF PH AND SALT CONCENTRATION COMBINED

A series of experiments was performed to test the effect of various salt concentrations combined with different H-ion concentrations. Of all these experiments, only one will be reported in detail. The experiment in question dealt with eight nutrient solutions of various NaCl content (3, 2, 1.5, 1.0, 0.8, 0.5, 0.25, 0.0 per cent, respectively), combined with six different H-ion concentrations (pH 7.6, 7.2, 6.6, 6.4, 6.2, 5.8). The results showed that at a NaCl concentration of from 3 to 0.8 per cent the H-ions have no visible effect, and no limited zone of growth appeared in any of these nutrient solutions. It was ascertained, on the other hand, that the NaCl concentration influences the density of population, if only slightly. It amounted at 3 per cent to 0.15, at 2 per cent to 0.3, at 1.5 per cent to 0.87, at 1 per cent to 1.5, and at 0.8 per cent to 1.8 amoebae per 0.1 cu. mm. nutrient solution. With the decrease of the NaCl concentration from 0.5 to 0.0 per cent, the H-ion concentration gained in importance. The latter's effect on the population maximum is clearly seen from Table IV.

These latter concentrations also showed clearly an effect upon the zone of growth. At 0.5 per cent NaCl and pH 7.6, 7.2, and 6.6 a diffuse growth, lacking a pronounced zone of growth, was observed; at pH 6.4 it was observed 5 mm. below the surface; at pH 6.2, 10

mm. below the surface; and at pH 5.8, 15 mm. below the surface. At NaCl concentration of 0.25 per cent, the first indistinct ring formation was observed at pH 7.2. At pH 6.6 a distinct one at a depth of 5 mm.; at pH 6.4, at a depth of 10 mm.; and at pH 6.2 and

TABLE IV

THE EFFECT OF DIFFERENT NaCl CONCENTRATIONS AND DIFFERENT H-ION CONCENTRATIONS COMBINED ON THE DENSITY OF POPULATION OF AMOEBAE

	pH					
	7.6	7.2	6.6	6.4	6.2	5.8
	0.5 per cent NaCl					
Amoebae	1.1	1.6	1.8	4.2	2.9	13.4
Cysts	1.1	1.4	2.4	5.2	6.1	14.5
Total	2.2	3.0	4.2	9.4	9.0	27.9
	0.25 per cent NaCl					
Amoebae	2.9	2.1	0.6	0.7	0.6	8.8
Cysts	1.3	3.5	4.6	9.3	12.1	38.6
Total	4.2	5.6	5.2	10.0	12.7	47.4
	0.0 per cent NaCl					
Amoebae	3.5	0.3	0.3	0.4	3.8	8.8
Cysts	0.8	3.5	8.1	5.8	21.4	26.9
Total	4.3	3.8	8.4	6.2	25.2	35.7

5.8, at a depth of about 15 mm. In the solution free of NaCl, a distinct zone of growth appeared already at pH 7.2, at a depth of 5 mm. In the presence of higher H-ion concentrations, the said zone developed uniformly at a depth of 15 mm.

Summarizing, we can state that in solutions of more than 0.5 per NaCl, the salt concentration has a decided influence upon the

growth, while the H-ion concentration has no effect. At low salt concentrations, however, the effects of both factors sum up.

EXPERIMENTS WITH VARIOUS DEXTROSE CONCENTRATIONS

These experiments proved conclusively that the depth of the ring of growth is not conditioned by the density of population in a purely mechanical way. The different dextrose concentrations attain dur-

TABLE V

THE EFFECT OF DIFFERENT DEXTROSE CONCENTRATIONS ON THE DENSITY OF POPULATION OF AMOEBAE

	Dextrose				
	0.0 per cent	0.5 per cent	1.0 per cent	2.0 per cent	4.0 per cent
Amoebae	0.2	0.1	0.3	0.0	0.0
Cysts	7.7	24.1	22.3	5.7	2.2
Total	7.9	24.2	22.6	5.7	2.2

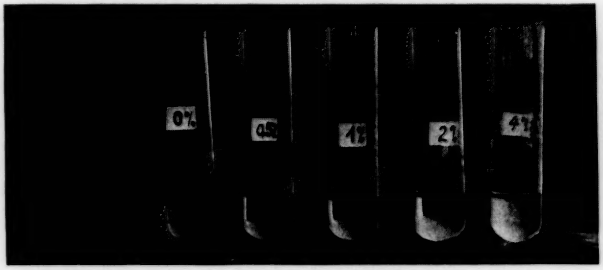

FIG. 3.—The different depths of the zones of growth at various dextrose concentrations.

ing the autoclaving of the nutrient solution various degrees of acidity. Therefore the solution was sterilized without dextrose and the desired concentration obtained by adding 0.5 cc. of a correspondingly diluted sterile dextrose solution. Concentrations of 0, 0.5, 1, 2, and 4 per cent, respectively, of dextrose were used. A uniform pH of 5.8 was thus obtained in all solutions. Table V and Figure 3 illustrate the results of these experiments clearly. The density of

population remains very low as well at 0 as at 2 per cent dextrose, and reaches its regular density in cultures at 0.5 and 1 per cent dextrose; the zone of growth, on the other hand, is equally deep in the first four concentrations and disappears only at 4 per cent dextrose. Thus, we observe a distinct ring formation at 0 and 2 per cent, despite the low density of population.

DISCUSSION

When discussing the results, one must naturally make a distinction between the problem of the attained effect on the maximal density of population and that of the effect on the depth of the zone of growth of amoebae. As far as the limits of the maximal population density are concerned, my results confirm the already previously known facts, which were recently found to hold true by Phelps (1935) also for sterile ciliate cultures. What factors determine this limitation is, however, not known yet. It is certainly not a matter of lack of nutritive material. We should rather assume a retarding effect of metabolic products on the division rate of the amoebae, which, under various external conditions, limit the propagation of the amoebae at different densities of population. Further research should aim at clearing up the nature of these division-preventing substances.

It is of interest to notice that some factors, such as NaCl concentration or pH, which affect the sterile cultures to a rather large extent are of only minor importance in cultures containing bacteria. Since the two cultures differ mainly by their manner of nutrition, the assumption that the foregoing factors injure the digestive apparatus of the amoebae, and principally their proteolytic capabilities, appears to be nearest the truth.

Secondly, the experiments show clearly that the amoebae are positive to different oxygen tensions under different external conditions. This makes it difficult to specify the oxygen requirement as a criterion for differentiating between the various species of amoeba. On the basis of the experiments here described, it is impossible to ascertain what factors condition the differences in behavior. The rate at which the once-disturbed stratification assumes its former state shows clearly that the amoebae aggregate actively around the

areas of optimal oxygen tension and that the ring formation consequently does not result from an increase in growth.

Also, the experiments do not ascertain whether the positive reactions to low oxygen concentrations is closely connected with the actual oxygen consumption of the cells and their manner of respiration.

SUMMARY

The effect of changes in the culture medium (pH, salt concentration, and dextrose concentration) upon the maximal density of population and the oxygen tension to which amoebae are positive has been studied. It was shown:

1. The maximal density of population decreases with increasing salt concentration as well as with increasing alkalinity.

2. The maximal density of population is highest at a dextrose concentration of 0.5–1 per cent.

3. In acid nutrient solutions and in those of lower salt concentrations the amoebae are positive to a lower oxygen tension than in alkaline solutions or those of higher salt concentration.

4. The concentration of dextrose from 0 to 2 per cent does not influence the reaction to oxygen tension.

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